Mechanism of Cornin in Preventing Astrocyte Autophagy Induced by Cerebral Ischemia/Reperfusion Injury

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Research Article

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Abstract

Background: Cornin is an iridoid glycoside isolated from the fruit of Verbena officinalis L, which has antioxidant activities and anti-ischemic effects. However, the underlying molecular mechanism of astrocytes autophagy induced by cerebral ischemia is still unknown. The purpose of this study was to investigate the effect of cornin against astrocytes autophagy induced by cerebral ischemia/reperfusion (CI/R) injury *in vitro* and *in vivo* and its potential mechanism.

Methods: Middle cerebral artery occlusion (MCAO) in rats and oxygen glucose deprivation/reperfusion (OGD/R) in U87 cells as a cerebral ischemia injury model *in vivo* and *in vitro*. Autophagy related protein, PI3K inhibitor LY294002, mTOR siRNA were used to evaluated the potential mechanism of cornin in autophagy.

Results: The results demonstrated that cornin significantly reduced the cerebral infarction volume and blood-brain barrier (BBB) leakage, and improved neurological recovery in middle cerebral artery occlusion (MCAO) rats. Cleaved caspase-3 and Bax levels were significantly decreased, while Bcl-2 and the apoptosis regulator ratio (Bcl-2/Bax) were markedly increased when treated with 2.5-10 mg/kg cornin. The obvious decreased expressions of Beclin-1 and LC3-II and increased of p62, phosphorylated protein kinase mTOR (p-mTOR), and phosphorylated Akt (p-Akt) were observed in MCAO rats treated with 2.5-10 mg/kg cornin, which was reversed by the PI3K inhibitor LY294002. In addition, cornin significantly increased NeuN and decreased GFAP, LC3-II, and Beclin-1 expression. The expression of autophagy-related proteins presented the same results as *in vivo* in oxygen glucose deprivation/reperfusion (OGD/R) in U87 cells. However, PI3K inhibitor LY294002 and mTOR siRNA reversed this result.

Conclusions: These results indicate that cornin improved neurological recovery after cerebral ischemia injury by preventing astrocytes autophagy induced by CI/R via the PI3K/Akt/mTOR signaling pathway.

Background

Stroke is an acute cerebrovascular condition that has high rates of morbidity, disability, and mortality [1]. Ischemic stroke is present in 80%-85% of all cases of stroke and can cause damage due to reperfusion that occurs in brain tissue. Currently, the most effective known method for stroke treatment is to give thrombolytic and neuroprotective agents [2]. However, the time window severely limits the scope of its use. In addition, no effective neuroprotective agent for the treatment of cerebral ischemia has been approved for market release in recent years.

Autophagy has an important function in cerebral ischemic injury, in which it causes progressive brain degeneration in animal and cellular models [3–5]. Autophagy activation occurs under special circumstances, particularly after the application of strong external stimuli such as intracellular damage due to hypoxia and ischemia, starvation, or viral infection. However, over activation of autophagy may result in cell death, which leads to autophagy-mediated or programmed cell death [6, 7]. Studies confirmed LC3-II and Beclin-1 levels were upregulated, whereas p62 was downregulated following

cerebral ischemia, consistent with an activation of autophagy [8]. Inhibiting autophagy activation can reduce ischemia injury and improve cell survival [8]. The atypical serine/threonine protein kinase mammalian target protein of rapamycin (mTOR) controls cell growth and proliferation [9], and is playing an important role in autophagy regulation. PI3K/Akt, a classical autophagy signaling pathway and the upstream signal of mTOR, plays an important regulatory role in mTOR activation [10]. Previous investigations have shown that mTOR-mediated signaling pathway phosphatidylinositol 3-kinase (PI3K)/RAC-α serine/threonine protein kinase (Akt)/mTOR controls autophagy in cerebral ischemia [11]. Furthermore, the activation of mTOR has a negative regulatory effect on autophagy. During the early phase of cerebral ischemia, injury stimulates the differentiation of astrocytes (AS), which protect the survival of neurons and help to maintain the normal function of the central nervous system. Astrocytes play an important role in neuroregulation and synaptic transmission [12]. Human U87 astrocytes classified as glioblastoma were employed as a cellular model in this study [13]. Therefore, astrocytes involved in autophagy through the PI3K/Akt/mTOR signaling pathway may be a potential treatment for ischemic stroke.

Cornin is an iridoid glycoside derived from the fruit of *Verbena officinalis L*. Previous studies confirmed that cornin has effects in lipid peroxidation inhibitory activity [14], cardioprotection against experimental myocardial ischemic injury [15], and brain protection against cerebral ischemic injury [16, 17]. However, the underlying molecular mechanism of brain protective effect during cerebral ischemia and reperfusion is unclear. The aim of the present study was to determine whether cornin could improved neurological recovery after cerebral ischemia injury by prevented astrocytes autophagy induced by CI/R, as well as the potential mechanism signaling, and if so, to determine its underlying neuroprotective mechanism using U87 cells and a cerebral ischemia/reperfusion (CI/R) rat model.

Materials And Methods

Drugs and reagents

Cornin (purity > 99.0%, molecular formula: $C_{17}H_{24}O_{10}$, molecular weight: 388.37) was obtained from SenBeiJia Biological Technology Co., Ltd. (Nanjing, China).

Antibodies against mTOR (Lot No: 2972S, 10), phospho-mTOR (Lot No: 2971S, 26), Akt (Lot No: 9272S, 28), phospho-Akt (Lot No: 13038S, 5), Bax (Lot No: 14796S, 2), and Bcl-2 (Lot No: 3498T,3) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against microtubule-associated proteins 1A/1B light chain 3B (LC3B) (ab48394, Lot No: GR3199111-5), cleaved caspase-3 (ab13585, Lot No: GR274345-1), Beclin-1 (ab62557, Lot No: GR3205839-5), LY294002 (PI3K inhibitor) (ab146593, Lot No: APN08061-6-12-S), p62 (ab56416, Lot No: GR3234289-1), and GFAP (ab7260, Lot No: GR3249186-1) were purchased from Abcam (Cambridge, UK). mTOR siRNA (sc-35409, Lot No: G0518) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Alexa Fluor 488-labelled anti-rabbit antibody (Abcam, UK, ab150077, Lot No: GR3245102-1) was used for immunofluorescence. Tetrazolium chloride (TTC) and

NeuN (MAB377, Lot No: 2919676) were obtained from Sigma (Shanghai, PR China), whereas Evans blue was bought from Urchem (Shanghai, PR China).

Animals

Male Sprague Dawley rats were obtained from Shandong Luye Pharmaceutical Company (Shandong, PR China, Permit Number: SCXK 2018 0003). The animals were each housed with the following conditions: 22°C ± 2°C, 50% ± 10% relative humidity, 12 h light/dark cycle, with food and water *ad libitum*. The Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80 − 23, USA), revised in 1996, was followed when conducting all animal studies. The studies were also carried out in compliance with the ARRIVE guidelines and the animal welfare compliance Guide for the Care and Use of Laboratory Animals (8thed., 2011). All experiments and procedures were conducted following the Animal Care Guidelines of the Animal Experiment Committee of Binzhou Medical University (China; authorization number BYLY 2015-74).

Induction of cerebral ischemia by ischemia/reperfusion procedure

After acclimatization for one week, the rats within the weight range of 270–300 g were injected intramuscularly with ketamine 100 mg/kg and xylazine 10 mg/kg to achieve anesthesia. The rectal temperature was kept constant throughout the surgical procedure, and the rectal temperature was recorded and maintained at 37°C. Middle cerebral artery occlusion (MCAO) was performed as described elsewhere [18]. In brief, occlusion of the left common carotid artery was performed, and then the branches of the external carotid artery were isolated and divided. The internal carotid artery was traced rostrally, and the 4 − 0 filament (a diameter of 0.25 mm), which had a tip diameter of 0.34 mm, was used to form a globular stopper and inserted into the internal carotid artery, and the filament was then pushed forward until resistance was observed. The filament was removed after 90 min.

Approximately 150 rats were divided into three groups, and the first two groups were further divided into five subgroups with 10 rats per group: I) non-ischemia/reperfusion (IR) (sham) group; II) I/R group (treated with saline only); III) 2.5 mg/kg cornin group; IV) 5 mg/kg cornin group; and V) cornin 10 mg/kg group. After reperfusion for 15 min, the indicated drugs were intravenously injected (*i.v.*) at various dosages via the tail vein. After 24 h of reperfusion, rats neurological deficit scores, as well as the blood-brain barrier (BBB) permeability were performed. The rats were placed in a clear glass box of approximately 10 L with a CO_2 perfusion rate of 2 liters per minute for euthanized. The brains were collected for brain infarct volume determination after transcardial perfusion with phosphate-buffered saline (PBS), the brains corresponding to the ischemic core, peri-infarct cortex rat brain tissues were collected and cutted into two pieces, one piece stored at -80°C and utilized in western blotting, the other piece was fixed with postfixed with 4% paraformaldehyde in PBS overnight at 4°C for subsequent immunohistochemical staining in the second group. For the mechanism study, 50 CI/R rats were divided into 5 groups: ⍰) non-ischemia/reperfusion (IR) (sham) group; ⍰) I/R group (treated with saline only); ⍰) 10 mg/kg cornin group; ⍰) 10 mg/kg cornin combined with LY294002 group; and ⍰) LY294002 group. After

24 h of reperfusion, the rats were euthanized with carbon dioxide (CO_2). The brains corresponding to the peri-infarct cortex were collected and utilized for Western blot analysis.

Assessment of Neurological Deficit Scores

Neurological deficit scores were assessed 24 h following reperfusion using the modified Neurological Severity Score (mNSS) method, and the 18-point method was employed to calculate the neurological function of rats. The scores were evaluated by an investigator who was blinded to the experimental treatment groups. The mNSS method includes a motion test, sensory test, balance beam test, reflex loss test, and abnormal exercise test [19].

Evans blue extravasation and measurement of infarct volume

At 24 h post-reperfusion, rats were sacrificed using deep anesthesia, and the brains were immediately collected. Five coronal sections of the brain with 2.0-mm thickness were prepared and incubated in a 2% TTC solution at 37°C for 20 min. Infarct brain tissues appear white, while normal brain tissues appear red. The sections were digitized, and the infarct areas were measured using Image Pro Plus software to calculate the percentage of cerebral infarct areas.

At 24 h post reperfusion, 0.1 ml of 4% Evans blue mixed in 0.9% saline was administered intravenously. A quantitative assay using Evans blue was performed to determine blood-brain barrier (BBB) leakage based on a method described previously [20].

Western blot analysis in vivo

Extraction of total protein from the ischemic penumbra in the cerebral cortex was performed in the experiment. The samples were homogenized with lysis buffer containing a protein inhibitor, the supernatant was obtained, and the amount of protein in the supernatant was determined using the BCA assay. Approximately 50 μg tissue protein samples were separated by SDS-PAGE and then analyzed by western blotting using specific antibodies against p-mTOR, mTOR, Bcl-2, Bax, p-Akt, Akt, Beclin-1, GFAP, p62, LC3B, cleaved caspase-3, and GAPDH at 4˚C overnight. A Gel Doc2000 system (Bio-Rad, USA) was used for scanning and quantification of strip optical density. The data were normalized to GAPDH.

PI3K inhibitor LY294002 intervention experiment

Rats were injected intramuscularly with ketamine 100 mg/kg (i.m.) and xylazine 10 mg/kg (i.m.) to achieve anesthesia. The CI/R rats were injected with LY294002 via the lateral ventricle approximately 30 min prior to reperfusion. LY294002 was dissolved in dimethyl sulfoxide (DMSO) immediately before the operation to obtain a final concentration of 10 mM, and 10 μl of LY294002 solution was injected into the rat's lateral ventricle using a brain stereotaxic device (Reward, Shenzhen, China) [21]. The stereotactic intracerebroventricular (ICV) injection site was selected from the following areas of the bregma: anterior and posterior, 0.8 mm; lateral, 1.5 mm; and depth, 3.5 mm.

Immunohistochemistry staining

The ischemic penumbra in the cerebral cortex was fixed in 4% paraformaldehyde for 48 hours, then dehydrated, embedded in paraffin, and sectioned at 4-μm thickness. The histological sections were placed on glass slides coated with polylysine, deparaffinized in xylene, and rehydrated across an alcohol gradient, then stained by immunohistochemistry. The expressions of NeuN, GFAP, LC3B, and Beclin-1 were detected. Positive expression was assessed using an Image-Pro Plus analysis system.

Cell culture

Human glioma U87 cells were obtained from the Cell Bank of the Chinese Academy of Sciences. The U87 cells were cultured in DMEM supplemented with 10% FBS and antibiotics consisting of 100 μg/ml streptomycin and 100 U/ml penicillin at a temperature of 37°C and 5% CO_2. The cells were subcultured upon reaching 80% confluency.

In vitro oxygen glucose deprivation/reperfusion model

To simulate *in vitro* glucose and oxygen deprivation, the U87 cells were subjected to a hypoxic environment for three hours. An *in vitro* oxygen glucose deprivation/reperfusion (*OGD/R*) model was established as in prior studies [22]. Before hypoxia, pretreatment of cells was performed using various cornin concentrations (10, 30, and 100 nM) for 48 h. A negative control was prepared using a normal culture using DMEM with 1% fetal bovine serum (FBS) in 5% CO_2 air and 20% O_2, whereas a hypoxia solution culture was employed as the control.

Cell viability assays

The U87 cells were kept in the hypoxic solution with or without cornin for three hours, and then cell viability was evaluated using an CCK-8 assay. Cells at a density of 1×10^5 cells/well were seeded into 96-well plates. The CCK-8 solution (10 μL/well) was placed into each well and further cultured at 37˚C for 4 hours. Absorbance at 450 nm was then assessed with a Molecular Devices SpectraMax M 5 instrument (Sunnyvale, CA, USA).

Western blot analysis in vitro

The U87 cells were cultured for 48 h, after which PI3K inhibitor LY294002 intervention was performed, and mTOR siRNA was added for transfection. This was followed by washing three times with ice-cold phosphate buffered sline (PBS) and lysing using RIPA lysis buffer (Beyotime, PR China) containing 1 mM PMSF (Sigma-Aldrich). Similar amounts of cell protein (30 μg) were separated by SDS-PAGE, and then analyzed by western blotting using specific antibodies against p-mTOR, mTOR, Beclin-1, p62, LC3B, p-Akt, and Akt, with β-actin as reference, at 4˚C overnight. The strips were scanned, and optical densities were quantified using a Gel Doc2000 (Bio-Rad, USA). Data normalization was performed using β-actin.

Determination of apoptosis

An Annexin-V FITC apoptosis detection kit was employed to identify apoptotic cells. First, the cells were collected, washed, and then cultured in the dark in the presence of Annexin-V FITC, as well as propidium

iodide, at 4°C for 30 min, followed by analysis using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Immunofluorescence detection

The distribution and expression of LC3B were detected by immunofluorescence in U87 cells. After cell treatment, imaging was conducted under a fluorescence microscope (Olympus IX73, Japan).

Statistical analysis

All experiments were performed in triplicate. The data from the experiments were expressed as the mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. Differences with $P < 0.05$ were considered statistically significant.

Results

Effects of cornin on neurological deficits, infarct volume, and BBB leakage

There were 5, 3, 3 and 2 and 2 rats (MCAO, cronin 2.5, 5 and 10 mg/kg groups, respectively) found died 24 h after the MCAO. Behavioral assessment is an important index for the recovery of neurological function. The mNSS scores in the MCAO group were higher compared with the sham operation group, whereas the scores were significantly ($P < 0.01$) reduced in cornin-treated rats at doses of 2.5, 5 and 10 mg/kg (Table 1).

Table 1
Effect of cornin on neurological deficits post CI/R.

Group	Survival rate	Infarct volume (%)	Neurological scores
Sham	20/20	0 ± 0	1.8 ± 0.57
MCAO	15/20	30.6 ± 4.5$^{\#\#}$	15.1 ± 2.8$^{\#\#}$
Cornin (2.5 mg/kg)	17/20	28.3 ± 3.0	12.7 ± 3.8
Cornin (5 mg/kg)	17/20	25.6 ± 5.3	11.0 ± 2.8*
Cornin (10 mg/kg)	18/20	20.8 ± 4.5**	10.4 ± 2.8**

Data are presented as the mean ± SD. Comparison of neurological deficit scores among groups using Kruskal Wallis followed by Dunn's test. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* MCAO group; $^{\#\#}P < 0.01$ *vs.* Sham group

The infarct volume was decreased in cornin-treated groups at doses of 2, 5, and 10 mg/kg in a dose-dependent manner, and was significantly decreased at 10 mg/kg compared to the MCAO group ($P < 0.01$) (Fig. 1A). Ischemia damaged the function of the BBB, which significantly increased the permeability of

the BBB in MCAO rats. BBB leakage was significantly ($P < 0.01$) decreased in the 10 mg/kg cornin group compared with rats in the MCAO group (Fig. 1B). These findings indicate that cornin alleviates CI/R injury.

Effect of cornin on the expression of apoptosis- and autophagy-related proteins in ischemic brain tissues through the PI3K/Akt/mTOR pathway

To detect the effects of cornin on autophagy and apoptosis after CI/R injury in rats, the Bcl-2, Bax, cleaved caspase-3, LC3-II, Beclin-1, and p62 protein levels were assessed. Western blotting revealed that the CI/R group exhibited significantly upregulated protein expression levels ($P < 0.05$) of Bax, cleaved caspase-3, LC3-II, and Beclin-1, and significantly ($P < 0.01$) decreased the expression levels of Bcl-2 and p62 compared with sham group. However, Bax, cleaved caspase-3, LC3-II, and Beclin-1 levels were significantly ($P < 0.01$) downregulated, while Bcl-2 and p62 were significantly ($P < 0.01$) upregulated in CI/R rats treated with cornin (Fig. 2 and Fig. 3). However, the expression of Bax and Bcl-2 was reversed by the PI3K inhibitor LY294002 (Fig. 5B). The expression of p-mTOR and p-Akt was markedly reducerd ($P < 0.01$, compared with sham group) after CI/R, and significantly ($P < 0.01$, compared with CI/R group) increased with cornin treatment (Fig. 3).

Effect of PI3K inhibitor LY294002 on the expression of autophagy-related proteins in ischemic brain tissues

To further verify whether cornin affects autophagy induced by CI/R injury through the PI3K/Akt/mTOR signaling pathway, PI3K inhibitor LY294002 was used in cornin and CI/R groups. Western blotting revealed that the expressions of p-mTOR, p-Akt, and p62 were all partially blocked by LY294002, whereas LC3-II and Beclin-1 were increased abundantly (Fig. 4). The results show that inhibiting the expression of PI3K also inhibits mTOR and activated excessive autophagy of the cells. Therefore, cornin may reduces the autophagy induced by CI/R injury through PI3K/Akt/mTOR pathway.

Protective effects of cornin on cerebral ischemia-reperfusion (CI/R) in immunohistochemistry-related protein

The expressions of NeuN, GFAP, LC3-II, and Beclin-1 at 24 h after CI/R injury were measured by immunohistochemistry. The expression of GFAP was lower in the sham operation group, and brown astrocytes were readily observed in the model group. Compared with the CI/R group, the number of positive astrocytes significantly decreased in cornin treated group ($P < 0.01$). The PI3K inhibitor LY294002, combined with cornin, significantly increased the number of positive astrocytes ($P < 0.05$) (Fig. 5A). At the same time, the expression of GFAP protein was detected by western blotting. The GFAP expression was significantly reduced after cornin treatment (compared with CI/R group, $P < 0.01$). Also, the PI3K inhibitor LY294002, when combined with cornin, significantly increased the expression of GFAP protein ($P < 0.05$) (Fig. 5B).

In the CI/R group, most of the neurons showed nuclear pyknosis, which is an indicator of injury induced by CI/R that was rarely observed in the cornin group, due to the neuroprotective effect of cornin. However, PI3K inhibitor LY294002 could decrease the protective effect of cornin ($P < 0.05$). The number of positive cells for Beclin-1 and LC3-II in the CI/R group was higher than that in the sham group, and the number of positive cells in the cornin group was significantly decreased compared to the CI/R group ($P < 0.01$). The combination of LY294002 and cornin increased the number of positive cells ($P < 0.05$) (Fig. 5A).

Effects of cornin on oxygen glucose deprivation/reperfusion induced cell death in U87 cells

Cornin also altered the course of oxygen glucose deprivation/reperfusion (OGD/R)-mediated U87 cell death. To evaluate the influence of cornin on the proliferation of U87 cells after OGD/R injury, the effect of 48-hour cornin treatment (10, 30, and 100 nM doses) on the viability and morphological changes in OGD/R-damaged U87 cells was assessed. Compared with normal cultured cells, the viability of the U87 cells treated with OGD/R was significantly inhibited (Fig. 6). However, following treatment with cornin (10, 30, and 100 nM), the viability of cells treated with OGD/R significantly increased in a concentration-dependent manner, and decreased at doses above 100 nM (Fig. 6).

Cornin inhibits OGD/R-induced U87 cell apoptosis and autophagy

Apoptosis in U87 cells was assessed by flow cytometry using AnnexinV-FITC/PI double staining, which included early apoptosis (Annexin V-FITC-positive, PI-negative) as well as late apoptosis (Annexin V-FITC-positive, PI-positive). The proportion of apoptotic cells in the model group was markedly higher than that observed in the normal control group, whereas the proportion of apoptotic cells in the cornin treatment group was reduced in a concentration-dependent manner (Fig. 7).

To confirm the protective effect of cornin on OGD/R-treated U87 cells, the effect of cornin on U87 cell autophagy was evaluated. Autophagy-associated protein LC3-II revealed that OGD/R significantly upregulated the level of LC3-II compared with the level observed in the normal control group. Cornin significantly inhibited the autophagy activation that was induced by OGD/R, which was demonstrated by the decrease in LC3-II expression (Fig. 8).

Cornin affects OGD/R-induced autophagy by activating the PI3K/Akt/mTOR pathway

To further verify whether cornin exhibited its effect on autophagy through the PI3K/Akt/mTOR signaling pathway, LY294002 (a PI3K inhibitor) was used in the cornin and OGD/R-treated groups. The protein expression levels of p-mTOR, p-Akt, and p62 significantly decreased after treatment with LY294002, whereas the protein expression levels of LC3-II and Beclin-1 significantly increased (Fig. 9). The application of mTOR siRNA induced a similar effect on the PI3K inhibitor LY294002. After mTOR siRNA transfection, the expression of mTOR and p62 significantly decreased, whereas LC3-II expression

significantly increased (Fig. 9). In summary, the results suggest that the astrocyte autophagy inhibitory effect of cornin involves the PI3K/Akt/mTOR pathway.

Discussion

In previous study, we have confirmed that cornin exerts a protective effect in CI/R injury, and its mechanism involves antioxidation and promoting angiogenesis effects [17]. In the present study, we determined that cornin significantly reduced the cerebral infarct volume, BBB leakage and improved functional recovery after CI/R injury in SD rats. The underlying mechanism of action is cornin prevented astrocytes autophagy induced by CI/R via the PI3K/Akt/mTOR signaling pathway.

The BBB is composed of vascular endothelial cells, basement membrane, podocytes and pericytes of astrocytes, microglia, as well as extracellular matrix. Among them, the tight connection between astrocytes and endothelial cells is the basis for the formation of BBB. Astrocytes also play an important role in neuroregulation and synaptic transmission [12]. Autophagy of astrocytes can be robustly activated by oxygen and glucose deprivation or pharmacological inhibition of metabolic stress induced by mTOR [23]. Furthermore, CI/R injury is strongly associated with autophagy process [24]. Studies have confirmed that inhibition of autophagic activity could attenuates CI/R injury [25]. The present study revealed that cornin play protective effects in both CI/R-induced autophagy in rats and OGD/R-induced autophagy in U87 cells. *In vitro*, excessive activation of autophagy by OGD/R decreases cell viability, whereas cornin inhibits the over activation of autophagy in a concentration-dependence manner. The increased LC3-II and Beclin-1 level in U87 cells and brain tissues were reduced and the level of p62 increased with cornin treatment. In addition, the protective effect of cornin was reversed by the autophagy activator LY294002 [26], and LC3-II and Beclin-1 expression were significantly increased. However, p62 decreased after LY294002 treatment. Similarly, the application of mTOR siRNA induced a similar effect on the PI3K inhibitor LY294002. *In vivo*, 10 mg/kg cornin significantly decreased infarct volume, and significantly decreased BBB leakage after CI/R injury. The mNSS scores behavioral assessment is also indicated cornin could markedly improve the recovery of neurological function.

Autophagosomes develop to enclose damaged organelles as well as other macromolecular structures [27]. However, the excessive formation of autophagosomes may aggravate cell injury and lead to autophagic cell death or apoptosis. In the case of CI/R, inflammatory factors secreted by brain tissues activate caspase-3, thereby leading to apoptosis [28]. Pro-apoptotic genes belonging to the Bcl-2 family, including Bax, can similarly activate caspase-3 and induce apoptosis [29]. Autophagy have tightly linked with apoptosis, and the anti-apoptotic protein Bcl-2 is also involved in the autophagy pathway [30]. In addition, Bcl-2 and Beclin-1 interact to target the Beclin-1-dependent autophagy pathway [31]. Our results revealed that the upregulation of cleaved caspase-3 and Bax in brain tissues was effectively reversed by cornin treatment, thereby leading to a marked increase in the Bcl-2 to Bax ratio. These effects were reversed by PI3K inhibitor LY294002. Therefore, cornin may exert its protective effects by inhibiting the apoptosis induced by CI/R. The protective effect of cornin on apoptosis could be associated with a reduction in autophagy as induced by CI/R.

During the autophagy process, the cytoplasmic form of LC3-I is transformed into phosphatidylethanolamine, which forms LC3-II, which in turn promotes the formation of autophagosomes. Therefore, the activation of autophagy can be expressed by an increase of LC3 expression [32]. Beclin-1 is an autophagy-associated protein as well as an important indicator of autophagy [33, 34]. To elucidate the mechanism by which cornin regulates autophagy, the effect of cornin on PI3K/Akt/mTOR pathway activation was evaluated. mTOR is a major regulator of cell growth, proliferation, and autophagy, as well as an important downstream target of the PI3K/Akt signaling pathway [35]. In ischemia and hypoxia, the supply of oxygen and nutrition is insufficient, and the level of ATP in the brain is reduced, which in turn inhibit the expression of mTOR and enhance autophagy. Previous studies have shown that PI3K/Akt/mTOR signaling is essential to the survival of neurovascular units in ischemia [36]. Our study has revealed that the infarct volume, as well as the leakage rate of the BBB, was significantly decreased, the functional recovery was significantly improved in rats after cornin treatment. These findings confirm that cornin alleviates CI/R injury. Simultaneously, cornin treatment markedly increased p-Akt and p-mTOR expression in U87 cells and brain tissues, and the PI3K inhibitor LY294002 significantly reduced the overexpression of p-Akt and p-mTOR. The increased LC3-II and Beclin-1 expression levels in U87 cells and brain tissues were reduced, and the expression level of p62 increased with cornin treatment. However, these effects were reversed by PI3K inhibitor LY294002. Similarly, the application of mTOR siRNA induced a similar effect on the PI3K inhibitor LY294002. These observations indicate that cornin contributes to the regulation of autophagy caused by CI/R through the PI3K/Akt/mTOR signaling pathway.

In summary, we have demonstrated that cornin plays neuroprotective effect after cerebral ischemia injury in SD rats by preventing astrocytes autophagy induced by CI/R via the PI3K/Akt/mTOR signaling pathway. The astrocytes were protected by autophagy inhibition of cornin, played an important effects on maintaining the integrity of BBB and also regulating neuroregulation and synaptic transmission of nerve cells [12]. A cascade of inflammatory injuries usually occur after cerebral ischemia injury. Whether cornin have anti-inflammatory effect and its mechanism in CI/R will be carried out in future.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Haibo Zhu and Shuping Zhang designed the experiments. Tianchi Lan, Yangyang Xu, Shucui Li performed the experiments. Ning Li analyzed the data. Tianchi Lan and Haibo Zhu wrote the paper. Shuping Zhang revised the paper. The author(s) read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

All the experimental protocols were in accordance with the guidelines of Binzhou Medical University and approved by Animal Experiment Committee of Binzhou Medical University (China; authorization number BYLY 2015-74). The Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, USA), revised in 1996, was followed when conducting all animal studies. The studies were also carried out in compliance with the ARRIVE guidelines and the animal welfare compliance Guide for the Care and Use of Laboratory Animals (8thed., 2011).

Consent for publication

Not applicable.

Competing interests

All authors declare that there are no competing financial or patent interests.

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Figures

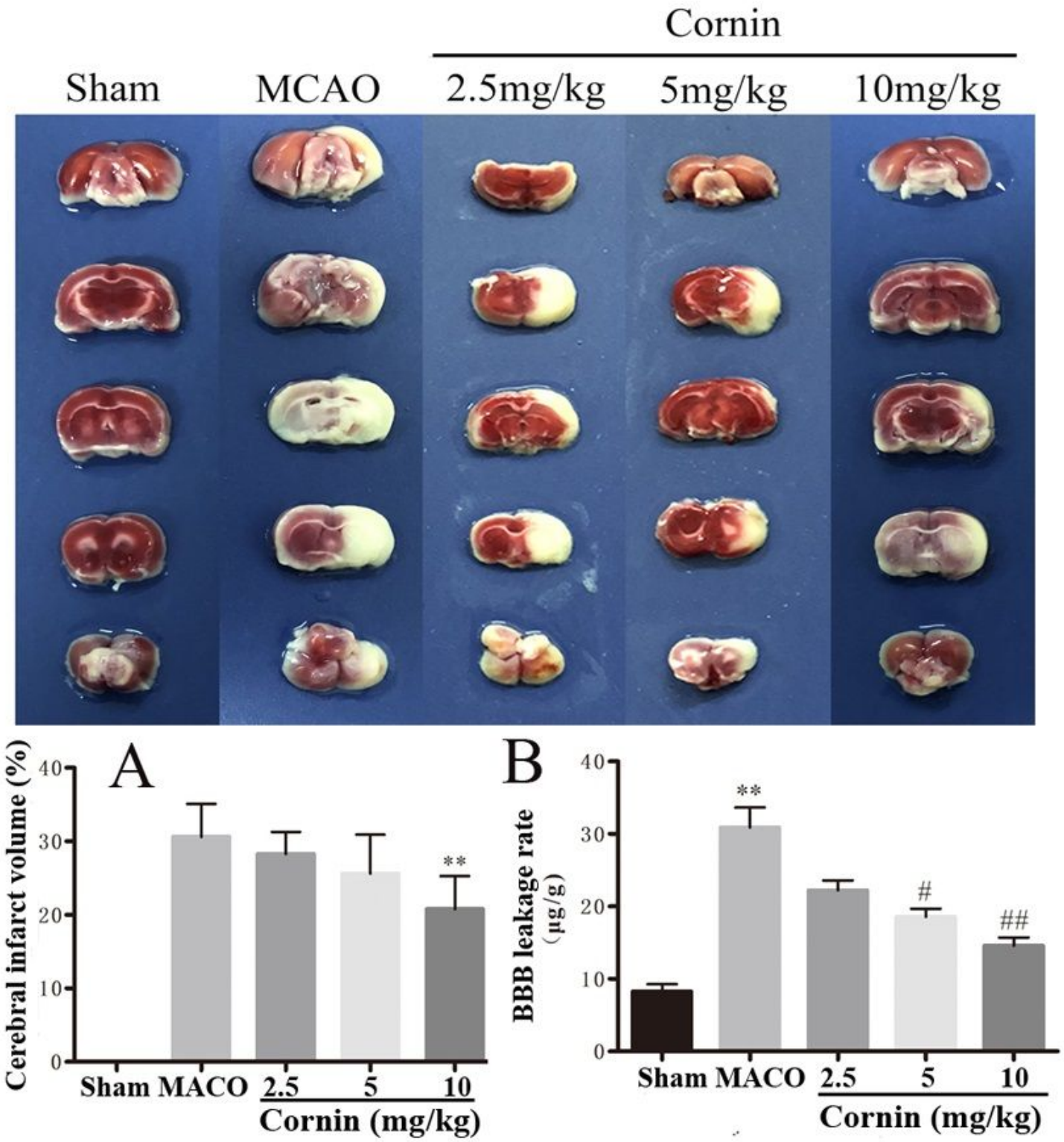

Figure 1

Effects of cornin on infarct volume and leakage in the blood-brain barrier. (A) Representative images of 2,3,4-triphenytetrazolium-chloride (TTC) staining and relevant quantitative analysis. The normal brain area appeared red, while the infarcted area was white. Quantitative analysis of cerebral infarct areas; the data are expressed as the mean ± SD, (n=6). $^{**}P < 0.01$ *vs.* MCAO group. **(B)** The permeability of the

blood-brain barrier was quantitatively assessed by measuring the extravasated Evans blue. The data are expressed as the mean ± SD, (n=6). $^{**}P < 0.01$ *vs.* the sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* MCAO group.

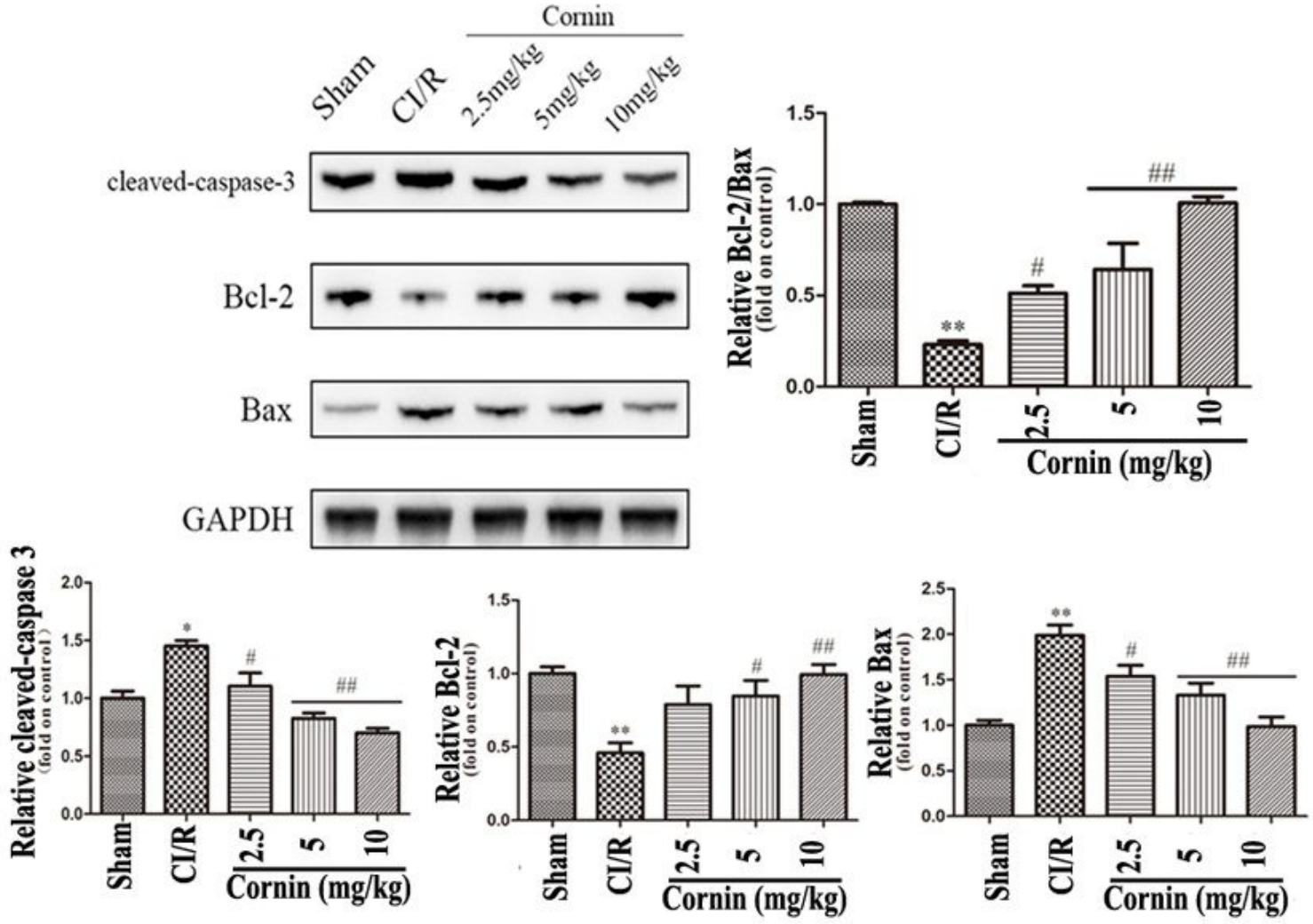

Figure 2

Effect of cornin on apoptosis in post cerebral ischemia-reperfusion (CI/R). Apoptosis-associated proteins were evaluated using western blot analysis, and the full-length blots/gels are presented in Figure S1. After CI/R, rats were treated with cornin (2.5, 5, 10 mg/kg) for 24 h, and equal amounts of tissue lysate protein (50 μg) were separated by SDS-PAGE and analyzed by western blotting using specific antibodies. The expression levels of cleaved caspase-3, Bcl-2, and Bax were determined and presented as the mean ± SD, (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* CI/R group.

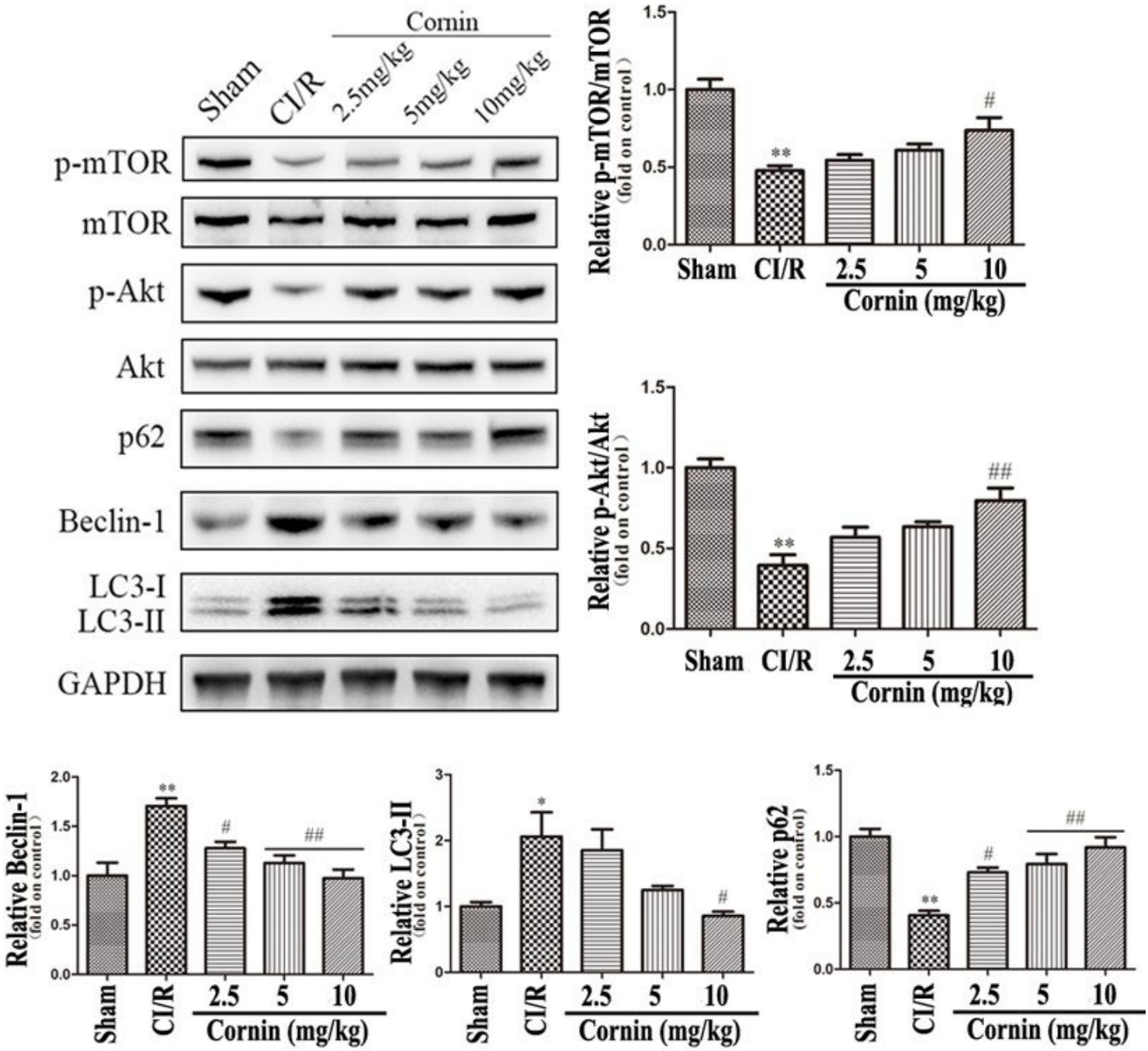

Figure 3

Effect of cornin on autophagy post cerebral ischemia-reperfusion (CI/R) through the PI3K/Akt/mTOR signaling pathway. Autophagy-associated proteins were assessed by western blotting using western blot analysis, and the full-length blots/gels are presented in Figure S2. After CI/R, rats were treated with cornin (2.5, 5, 10 mg/kg) for 24 h, and equal amounts of tissue lysate protein (50 μg) were separated by SDS-PAGE and analyzed by western blotting using specific antibodies. LC3-II, p62, Beclin-1, p-mTOR, and p-Akt expression levels were quantified and are represented as the mean ± SD, (n=3). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* CI/R group.

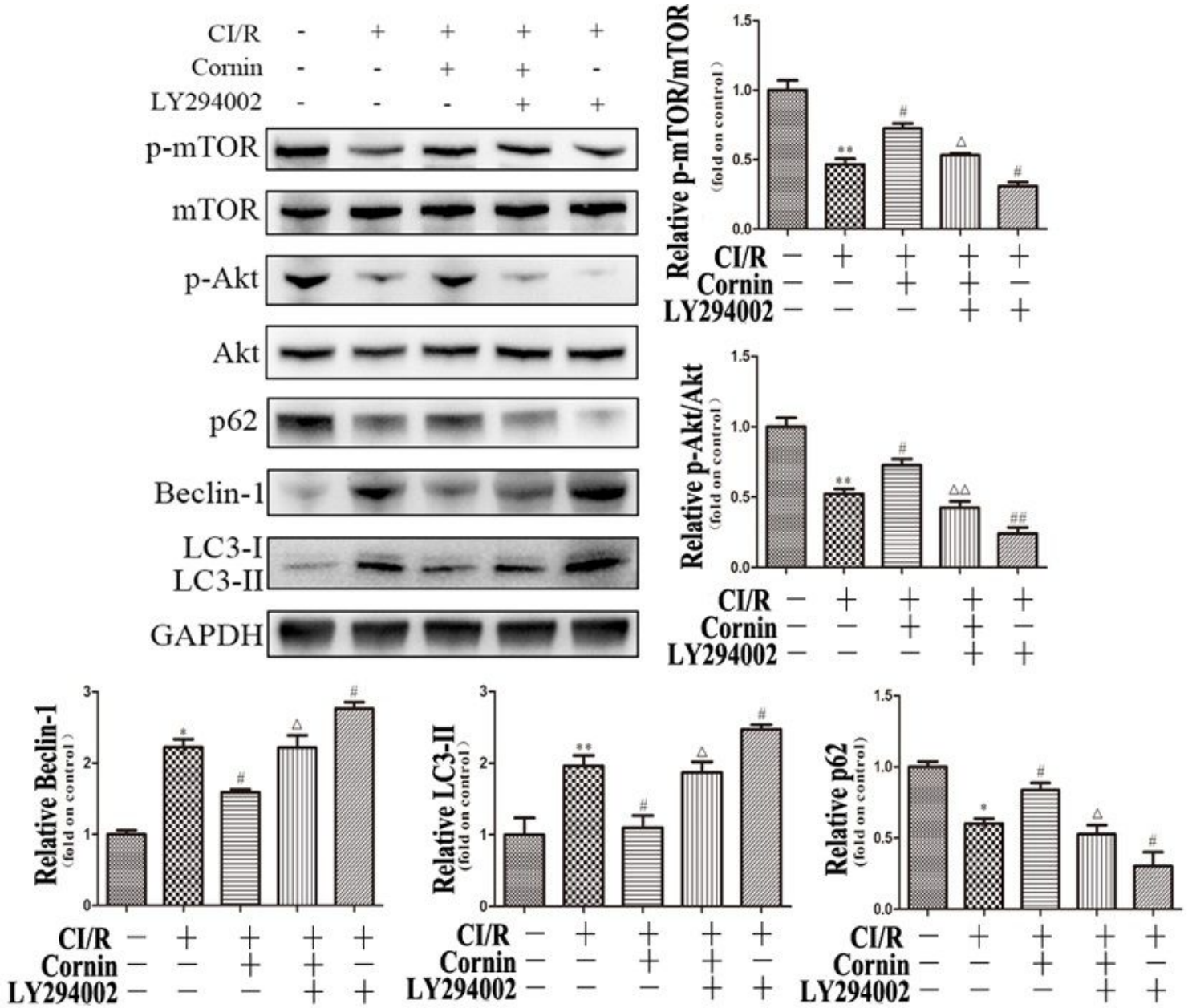

Figure 4

Effect of PI3K inhibitor LY294002 on autophagy post cerebral ischemia-reperfusion (CI/R). The rats were injected with PI3K inhibitor LY294002 (10 mM) before cornin (10 mg/kg) treatment. Autophagy-associated proteins were assessed by western blotting using western blot analysis, and the full-length blots/gels are presented in Figure S3. LC3-II, p62, Beclin-1, p-mTOR, and p-Akt expression levels were quantified and are represented as the mean ± SD, (n=3). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* CI/R group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ *vs.* cornin 10 mg/kg group.

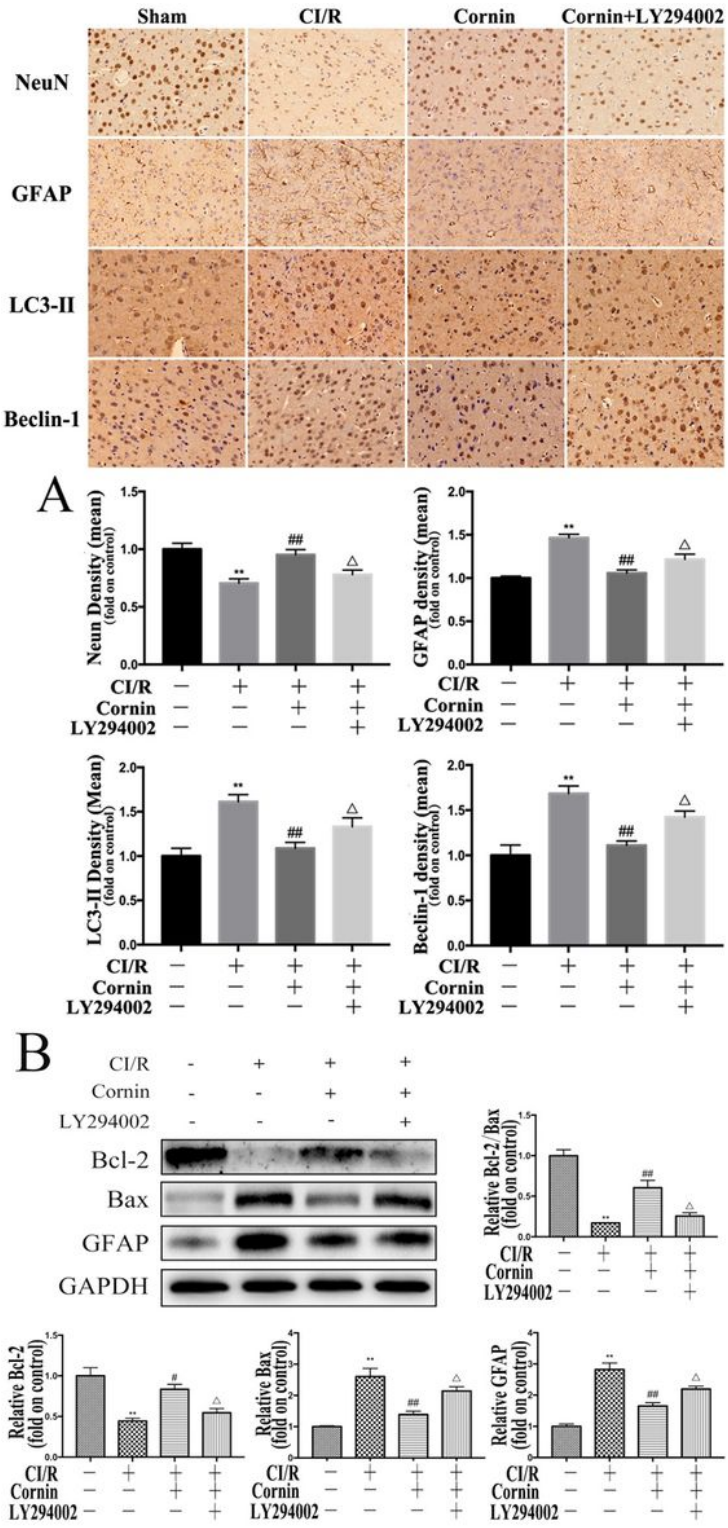

Figure 5

Protective effects of cornin on cerebral ischemia-reperfusion (CI/R) assessed by immunohistochemistry and western blot. (A) After 24 hours of CI/R injury, the number of cells positive for NeuN, GFAP, LC3-II, and Beclin-1 in the sham group, CI/R group, cornin (10 mg/kg) group, and cornin + LY294002 group was quantitatively evaluated by immunohistochemistry. The magnification was ×200. The data are presented as the mean ± SD, (n = 3). $^{**}P < 0.01$ *vs.* the sham group; $^{\#\#}P < 0.01$ *vs.* CI/R group; $^{\Delta}P < 0.05$ *vs.* cornin 10

mg/kg group. **(B)** GFAP, Bax and Bcl-2 was assessed by western blotting using western blot analysis, and the full-length blots/gels are presented in Figure S4, and the expression levels were quantified and are represented as the mean ± SD, (n = 3). $^{**}P < 0.01$ *vs.* the sham group; $^{\#\#}P < 0.01$ *vs.* CI/R group; $^{\Delta}P < 0.05$ *vs.* cornin 10 mg/kg group.

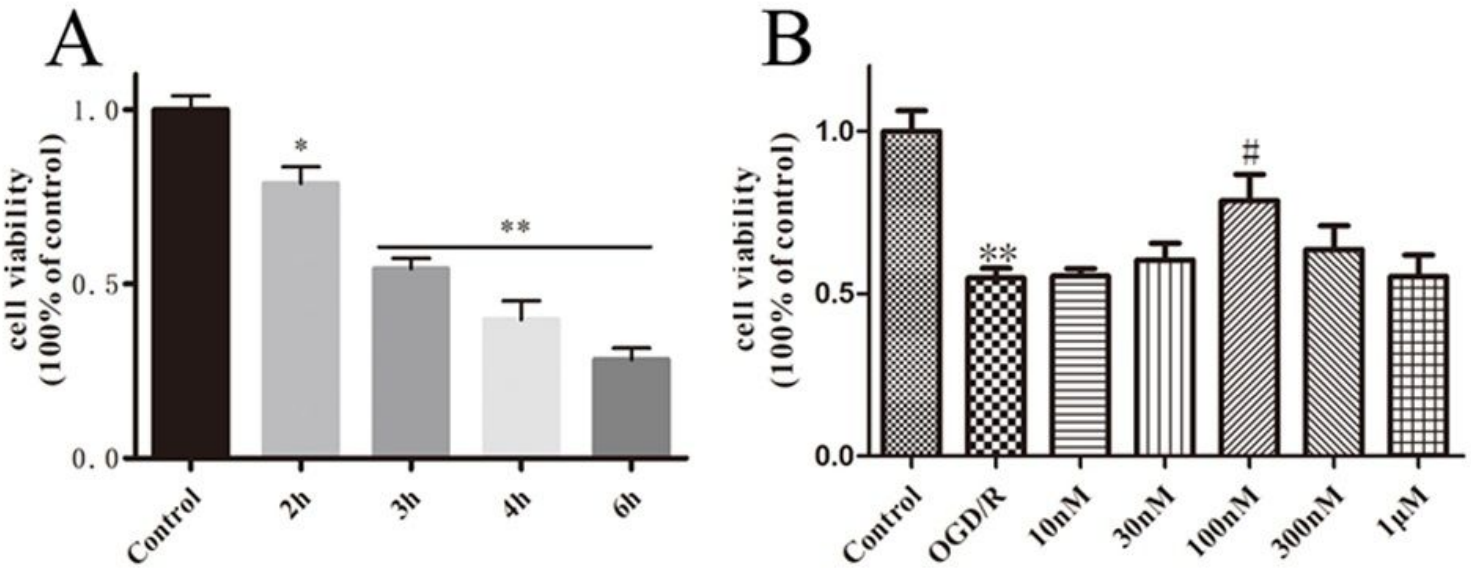

Figure 6

Protective effect of cornin in OGD/R-induced U87 cell death. (A) The viability of U87 cells exposed to OGD/R at different durations as assessed using the CCK-8 assay. **(B)** U87 cells were pre-incubated with different concentrations of cornin for 48 h, then underwent oxygen-glucose deprivation for 3 h, followed by reoxygenation for 24 h to detect cell viability changes. Data are expressed as the mean ± SD, (n = 6). Significance was assessed by a one-way ANOVA followed by Dunnett's test. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the control group; $^{\#}P < 0.05$ *vs.* the OGD/R group.

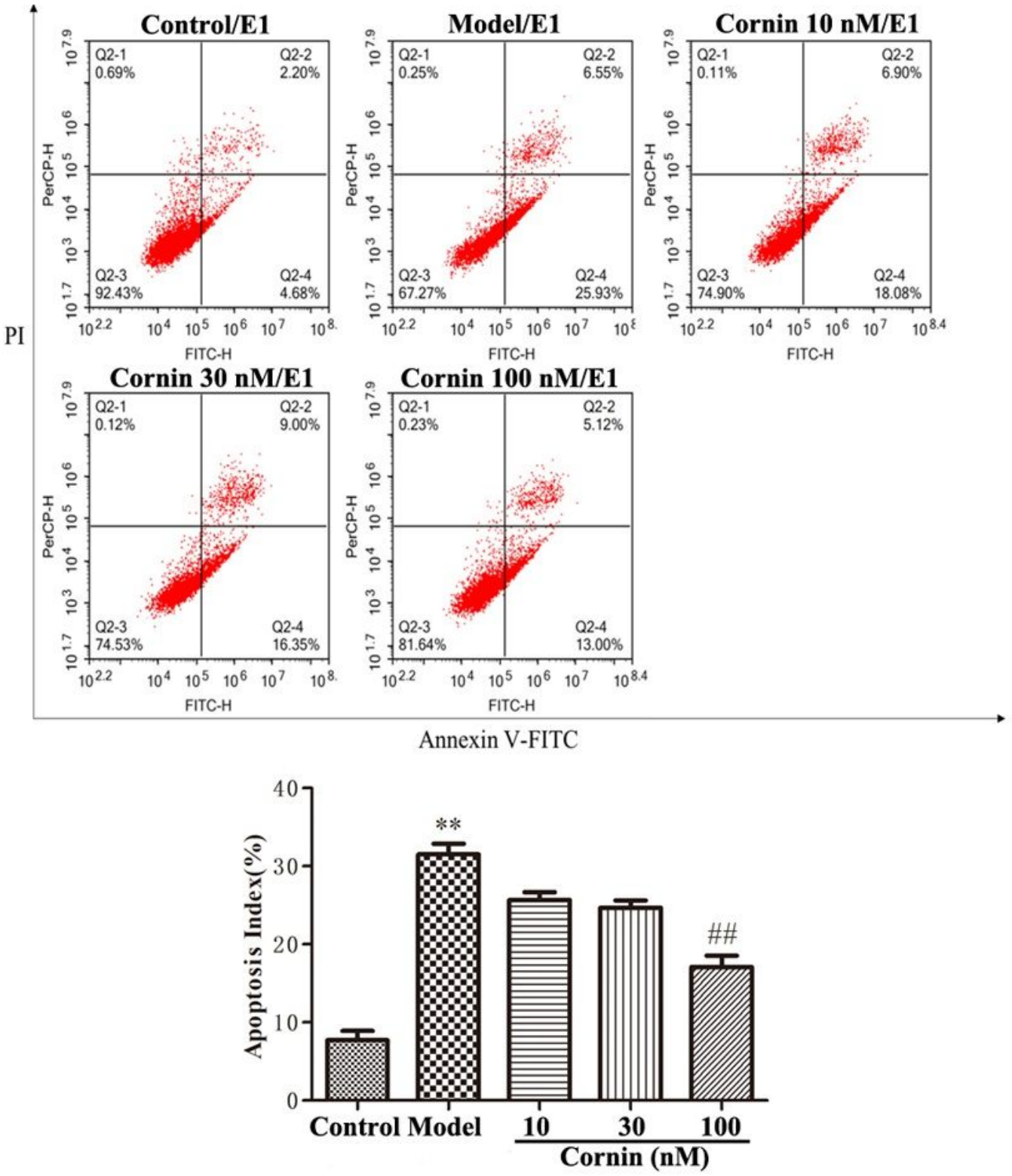

Figure 7

Effect of cornin on U87 cell apoptosis. U87 cells were pretreated with cornin (10, 30, 100 nM) for 48 hours, followed by oxygen-glucose deprivation exposure for three hours, and reoxygenation for 24 h. Apoptosis was assess by flow cytometry using Annexin V-FITC/PI fluorescence staining. The sum of the second and fourth quadrants in the figure is the percentage of apoptotic cells. The data of apoptotic cells are expressed as the mean ± SD, (n = 3). $^{**}P < 0.01$ *vs*. the control group; $^{\#\#}P < 0.01$ *vs*. the OGD/R group.

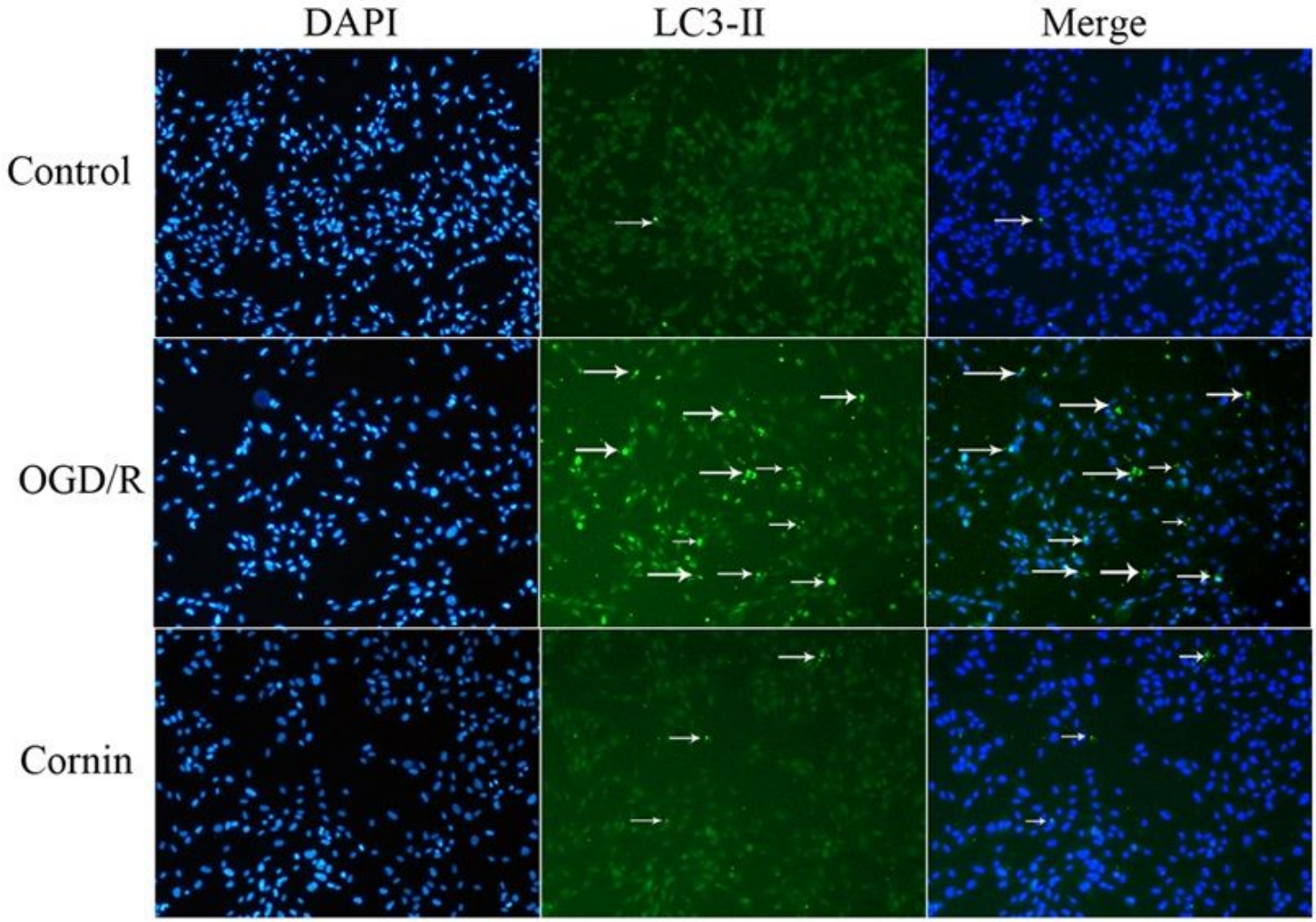

Figure 8

Effect of cornin on U87 cell autophagy. U87 cells were pretreated with cornin (100 nM) for 48 hours followed by oxygen-glucose deprivation exposure for three hours, and reoxygenation for 24 h. Immunofluorescence images of U87 cells using the autophagy marker LC3B. The left column shows a nucleus stained with DAPI, the middle column presents immunofluorescence staining using specific markers, and the right column depicts a merged image of the left and middle frames.

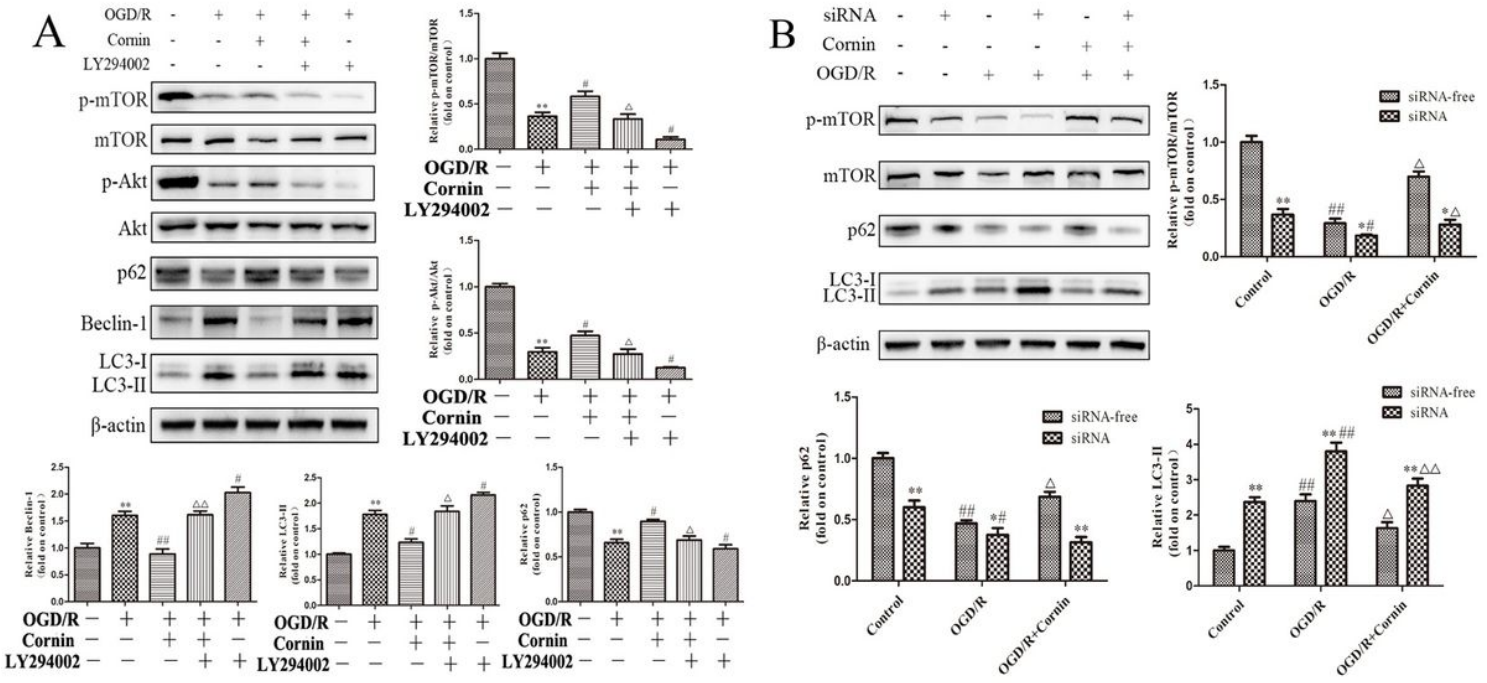

Figure 9

Effect of cornin on autophagy in OGD/R-induced U87 cells through the PI3K/Akt/mTOR signaling pathway. (A) LY294002 (10 mM), a PI3K inhibitor, was used as pretreatment for 6 h, and then incubated with cornin (100 nM) for 48 h. Western blotting of the associated proteins and density analysis were performed, and the full-length blots/gels are presented in Figure S5.. The results are presented as the mean ± SD, (n = 3). $^{**}P < 0.01$ vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* the OGD/R group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ *vs.* 100 nM cornin group. **(B)** U87 cells were pretreated using mTOR siRNA for 6 hours followed by western blotting using western blot analysis, and the full-length blots/gels are presented in Figure S6. The results are presented as the mean ± SD, (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* siRNA-free treatment group; and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* the control group, respectively; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ *vs.* OGD/R group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryDatasetFile.pdf