Original Research

Apelin-13 prevents the effects of oxygen–glucose deprivation/reperfusion on bEnd.3 cells by inhibiting AKT–mTOR signaling

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Impact statement

Impairment of the blood–brain barrier (BBB) is a pivotal pathophysiological process related to cerebral ischemia/reperfusion (I/R) injury, but the underlying mechanisms are complex and remain uncertain. We hypothesized that apelin-13 protects cerebral microvascular endothelial cells against I/R injury by regulating autophagic activity. Thus, we examined whether apelin-13 protects cerebral microvascular endothelial cells, which are important components of the BBB, from I/R injury by regulating autophagy. The results showed that oxygen–glucose deprivation/reoxygenation (OGD/R) stress can induce autophagic activity in cerebral microvascular endothelial cells, and that apelin-13 protects tight junction (TJ) proteins in adjacent cells and cell migration capacity by upregulating autophagic activity induced by OGD/R injury. These observations support the idea that apelin-13 could be used to develop a novel therapeutic approach to I/R injury.

Abstract

Autophagy plays works by degrading misfolded proteins and dysfunctional organelles and maintains intracellular homeostasis. Apelin-13 has been investigated as an agent that might protect the blood–brain barrier (BBB) from cerebral ischemia/reperfusion (I/R) injury. In this study, we examined whether apelin-13 protects cerebral microvascular endothelial cells, important components of the BBB, from I/R injury by regulating autophagy. To mimic I/R injury, the mouse cerebral microvascular endothelia l cell line bEnd 3 undergoes the process of oxygen and glucose deprivation and re feeding in the process of culture. Cell viability was detected using a commercial kit, and cell migration was monitored by *in vitro* scratch assay. The tight junction (TJ) proteins ZO-1 and occludin; the autophagy markers LC3 II, beclin 1, and p62; and components of the AKT–mTOR signaling pathway were detected by Western blotting and immunofluorescence. To confirm the role of autophagy in OGD/R and the protective effect of apelin-13, we treated the cells with 3-methyladenine (3-MA), a pharmacological inhibitor of autophagy. Our results demonstrated that OGD/R increased autophagic activity but decreased viability, abundance of TJs, and migration. Viability and TJ abundance were further reduced when the OGD/R group was treated with 3-MA. These results indicated that bEnd.3 upregulates autophagy to ameliorate the effects of OGD/R injury on viability and TJs, but that the autophagy induced by OGD/R alone is not sufficient to protect against the effect on cell migration. Treatment of OGD/R samples with apelin-13 markedly increased viability, TJ abundance, and migration, as well as autophagic activity, whereas 3-MA inhibited this increase, suggesting that apelin-13 exerted its protective effects by upregulating autophagy.

Keywords: Apelin-13, autophagy, blood–brain barrier, pathway, cell migration, ischemia/reperfusion (I/R) injury

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Introduction

Stroke, one of the most common causes of death, contributes to the high incidence of disability around the world. Stroke is associated with significant health and economic impacts and consumes significant medical resources. In ischemic stroke, which accounts for about 80% of all strokes, cerebral ischemic injury is caused by sudden occlusion of blood vessels due to thrombus or embolism, leading to a shortage of normal energy and nutrients in brain tissue.[1,2] Because cerebral homeostasis and function rely on an adequate oxygenation, recovery of blood supply is essential to reverse cerebral ischemic injury. However, in addition to the damage caused by blockage of blood flow, reperfusion itself can induce many additional complications through secondary brain tissue injury.[3] Accordingly, the prevention and treatment of cerebral ischemia/reperfusion (I/R) injury are key strategies for stroke intervention. Impairment of the blood–brain barrier (BBB) is a pivotal pathophysiological process related to cerebral I/R injury,[4] but the underlying mechanisms are complex and remain uncertain.

The BBB, which consists of highly specialized microvascular endothelial cells, protects the central nervous system (CNS) from infections and toxins.[5] Within the BBB, specific proteins form close connections, called tight junctions (TJs), between adjacent endothelial cells. TJ proteins comprise

several integral membrane proteins and cytoplasmic accessory proteins such as claudins, occludin, junction adhesion molecules, and zonula occludens (ZO) proteins. Occludin, a four-transmembrane protein that is constitutively expressed in brain endothelial cells, is the main component of TJs and plays important roles in the stability and barrier functions of these structures.[6] ZO proteins are cytoplasmic factors that support TJs. TJ proteins play critical roles in maintaining the polarity of the barrier and also participate in cellular signal transduction. Loss of TJ proteins is associated with an increase in BBB permeability.[7] I/R disrupts TJ proteins in endothelial cells and thus perturbs the integrity of the BBB,[8] while the molecular mechanism underlying BBB breakdown following I/R remains elusive.

Autophagy is a catabolic process in which intracellular components are broken down in the lysosome. This process helps to keep a balance between synthesis, degradation, and subsequent recycling of cellular products.[9–11] Various stress conditions, including hunger, hypoxia, infection, aging, and oxidative stress, can activate autophagy.[12,13] A kinase complex consisting of class III phosphatidylinositol 3-kinase (PI3K), a myristoylated protein kinase, and beclin 1 is required for the initial nucleation and assembly of the primary autophagosome membrane. Two ubiquitin-like conjugated systems mediate further elongation of the isolation membrane, and one of them results in conversion of microtubule-associated protein 1 light chain 3 (LC3) from the free form (LC3 I) to a lipid-conjugated membrane-bound form (LC3 II).[14] Accordingly, accumulation of LC3 II is commonly used as a marker of autophagy. In addition, mammalian target of rapamycin (mTOR) inhibits the expression or activity of LC3 and beclin-1 proteins, and an abundance of nutrients activates mTOR, leading to suppression of autophagy; conversely, stress conditions such as starvation or hypoxia inhibit mTOR and activate autophagy.[15–17] Several lines of evidence show that autophagy plays important roles in regulating the disruption of TJ proteins and the integrity of BBB.[18] Moreover, autophagy is activated during cerebral I/R injury, and regulation of autophagy affects the results of cerebral I/R injury.[19–22] However, the molecular mechanism of autophagy involved in the induction of I/R injury has not been specifically described.

Apelin is the endogenous ligand for APJ, which is a G-protein-coupled receptor.[23] The basic signal transduction mechanism of apelin acting on APJ is determined by Gαi activation trigger.[24] Apelin acting on APJ can also cause Gαo and Gαq activation.[25] Apelin is synthesized as a precursor that is cleaved to yield a family of bioactive fragments, including apelin-36, apelin-19, apelin-17, apelin-13 (or its pyroglutamate analog, [Pyr] 1-apelin-13), and apelin-12, of which apelin-13 is the most bioactive.[26,27] APJ proteins and its endogenous ligand apelin are widely expressed in the CNS and peripheral tissues, with the highest levels observed in cerebellum, hypothalamus, vascular endothelium, heart, lung, and kidney.

The apelin/APJ system is involved in the regulation of various physiological functions and pathophysiological states, including fluid and glucose homeostasis, feeding behavior, angiogenesis, cell proliferation, and immunity.[28,29] Apelin protects the brain against I/R injury: in mice, apelin-13 ameliorates neurological defects, decreases brain infarct volume, and reduces brain edema after I/R injury.[27,30,31] In mechanistic terms, however, it remains unclear how apelin is involved in the resistance of I/R intervention. Therefore, we assumed that apelin-13 protects cerebral microvascular endothelial cells against I/R injury by regulating autophagic activity. In this study, we examined whether apelin-13 protects cerebral microvascular endothelial cells, important components of the BBB, from I/R injury by regulating autophagy.

Materials and methods

Culture of bEnd.3 cells

The bEnd.3 (BNCC Cell Bank, Beijing, China) is a line of immortalized cerebral microvascular endothelial cell line with key characteristics of the BBB phenotype including expression of TJ proteins such as ZO, claudins, and occludin [4, 24]. bEnd.3 cells were cultured as monolayers in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) which contained 10% fetal bovine serum (FBS, Gibco), 100 μg/mL streptomycin (Gibco), and 100 U/mL penicillin (Gibco). Cells were cultured at 37°C in an incubator containing 5% CO2.

Oxygen–glucose deprivation/reoxygenation cell model

An oxygen–glucose deprivation/reoxygenation (OGD/R) cell model was used to mimic I/R *in vitro*. To induce OGD/R injury, DMEM was replaced with glucose- and serum-free blocker buffer, and the cultures were held at 37°C for 4 h in a hypoxic incubator equilibrated with 95% N2 and 5% CO2. For reoxygenation, the cells were incubated for 16 h in their original medium (containing serum and glucose) in an incubator containing 70% N2, 25% O2, and 5% CO2.

Cytotoxicity assays

Cell viability was evaluated colorimetrically using the Cell Counting Kit-8 (CCK-8, Sigma-Aldrich, St. Louis, MO, USA). bEnd.3 cells were cultured on 96-well cell culture plates (Corning, Corning, NY, USA). Following OGD injury, cells were exposed to reoxygenation for various times (0, 4, 8, 16, or 24 h) in the presence of various concentrations of apelin-13 (10^{-11}–10^{-6}M) (Phoenix Pharmaceuticals, Belmont, MA, USA). After reoxygenation, 10 μL CCK-8 assay solution was added to 100 mL medium, and the cells were incubated for an additional 1 h. Optical densities (ODs) at 450 nm were measured on a microplate reader.

Immunofluorescence and confocal microscopy

bEnd.3 cells were grown in 12-well plates on glass cover slips pretreated with 0.1 mg/mL solution of poly-L-lysine (Sigma). Cell monolayers were fixed with precooled ethanol for 20 min after OGD/R injury carried out as described above. Non-specific binding was blocked by incubation in 3% BSA (phosphate buffered saline [PBS]) for 1 h at room temperature, and the slides were incubated overnight at 4°C in a humidified environment with antibodies against LC3 II (1:100, Cell Signaling Technology, Danvers, MA, USA)

and ZO-1 (1:30, Proteintech Group, Rosemont, IL, USA). Cell monolayers were then incubated for 1 h at 37°C with Cy3-labeled secondary antibody (1:100, Boster Biological Technology, Wuhan, China). Slides were examined under an oil immersion objective (63×) on a Leica DMRE laser scanning confocal microscope (Leica, Milton Keynes, UK).

Western blot assays

Cells were lysed with Radio Immunoprecipitation Assay lysis buffer (Beyotime, Shanghai, China) supplemented with Phenylmethanesulfonyl Fluoride and phosphatase inhibitor (Roche). Protein samples were analyzed by SDS-PAGE on 8–12% gels and transferred to Polyvinylidene Fluoride membranes at 4°C. Membranes were blocked with 5% skim milk in TrisBuffered Saline Tween20 for 2 h at room temperature, and then incubated at 4°C overnight with the following primary antibodies: anti-β-actin (1:5000, Zhongshan Golden Bridge, Beijing, China), anti-LC3 II (1:1000, Cell Signaling Technology), anti-p62 (1:1000, Cell Signaling Technology), anti-Beclin 1 (1:1000, Cell Signaling Technology), anti-phospho-AKT (1:1000, Cell Signaling Technology), anti-AKT (1:1000, Cell Signaling Technology), anti-phospho-mTOR (1:1000, Cell Signaling Technology), anti-mTOR (1:1000, Cell Signaling Technology), anti-ZO-1 (1:1000, Proteintech Group), and anti-occludin (1:1000, Proteintech Group). Membranes were washed three times for 15 min, incubated with anti-rabbit or anti-mouse secondary antibodies conjugated to Horseradish Peroxidase (1:5000, Zhongshan Golden Bridge), and then subjected to chemiluminescence assay using the Efficient Chemiluminescence reagent kit (MultiSciences, Hangzhou, China). OD was quantified using the ImageJ software.

In vitro scratch assays

In vitro scratch assays were performed to assess cell migration. Briefly, 5 × 105 bEnd.3 cells were seeded in six-well culture plates and cultured in DMEM with 10% FBS for 24 h until confluence reached 85–90%. Using a sterile ruler and a 200-μL pipette tip, a straight line was carefully scratched into the monolayer across the center of every well. A second straight line perpendicular to the first was scratched to create a cross-shaped gap. The pipette tip was kept perpendicular to the plate bottom throughout this process. Each well was washed twice with 1 mL PBS to remove any detached cells. OGD/R injury was carried out as described above, and digital images of the gaps were acquired at various time points (0 h [before injury], OGD4/R16, OGD4/R24, and OGD4/R36). Gap areas were quantitatively evaluated using cellSens Standard software (Olympus Life Sciences, Waltham, MA, USA). Healing rate = (OGD4h/R(n)h − OGD0h) / OGD0h × 100%.

Statistical analysis

Parametric data are presented as means ± SEM (standard error of mean). One-way analysis of variance (ANOVA) followed by Tukey test and Student's *t*-test (to compare the means of two groups) were performed using GraphPad Prism. $P < 0.05$ for two-tailed tests was considered statistically significant. The number of experimental samples used in each group is presented in the corresponding figure legend.

Results

Effect of OGD/R and apelin-13 on cell viability

To detect the effect of reperfusion on cell viability, we used the CCK-8 kit to detect changes in cell viability at various time points after 4 h of OGD (OGD4h/R(n)h: R0 h, R4 h, R8 h, R16 h, and R24 h). As shown in Figure 1(A), relative to the control group, mouse cerebral microvascular endothelial cell (bEnd.3) viability decreased significantly after OGD4/R0, whereas after 4 h of reoxygenation, cell viability gradually increased, but decreased after OGD4/R16. To calculate the EC50 (concentration for 50% maximal effect) of apelin-13, we evaluated cell viability following treatment with a range of concentrations (from 10^{-6} to 10^{-11} M) of apelin-13. As shown in Figure 1(B), the EC50 of apelin-13 was 4.171×10^{-9}M. We then tested the protective effect of apelin-13 on bEnd.3 after OGD4/R16. This treatment significantly decreased cell viability, but apelin-13 alleviated the effects of OGD/R injury and increased cell viability (Figure 1(C)).

Apelin-13 protects against OGD/R-induced disruption of TJ proteins and cell migration

In normal, extensive TJ proteins laying in the cytoplasmic membrane of adjacent endothelial cells and sealing the intercellular cleft with lines were seen (Figure 2(D)). OGD/R injury significantly decreased the expression of occludin and ZO-1. Because expression of ZO-1 was very low in the OGD/R samples, only a few junction points remained between adjacent endothelial cells, and no junction line was formed by fusion between ZO-1 molecules. Expression levels of occludin and ZO-1, as well as the number of junction points of ZO-1 between the cytoplasmic membranes of endothelial cells, were significantly higher after addition of apelin-13 to bEnd.3 cells injured by OGD/R (Figure 2(A) to (D)). Apelin-13 protected endothelial cell membrane junction proteins from OGD/R injury in a concentration-dependent manner, and expression of occludin and ZO-1 increased along with apelin-13 concentration (Figure 2(E) and (F)).

Migration is a critical component of endothelial cell angiogenesis. Hence, we next investigated the role of apelin-13 in migration. In a scratch wound-healing assay, the healing rate decreased significantly following exposure to OGD/R, but this reduction in healing rate was abolished by treatment with apelin-13 (Figure 2(G) and (I)). This implies that OGD/R injury decreases bEnd.3 migration and that apelin-13 protects against this effect.

Apelin-13 promotes OGD/R injury–induced autophagy

To explore the mechanism of OGD/R injury and the protective effect of apelin-13, we examined autophagy. Using Western blot assays, we analyzed the expression levels of autophagy-related marker proteins LC3 II, beclin 1, and p62. As shown in Figure 3(A) to (D), OGD/R induced a significant increase in the levels of endogenous LC3 II and beclin 1, as well as a significant decrease in the level of p62; in addition, when cells were treated with apelin-13 during OGD/R, LC3 II and beclin 1 were further upregulated and p62 was further

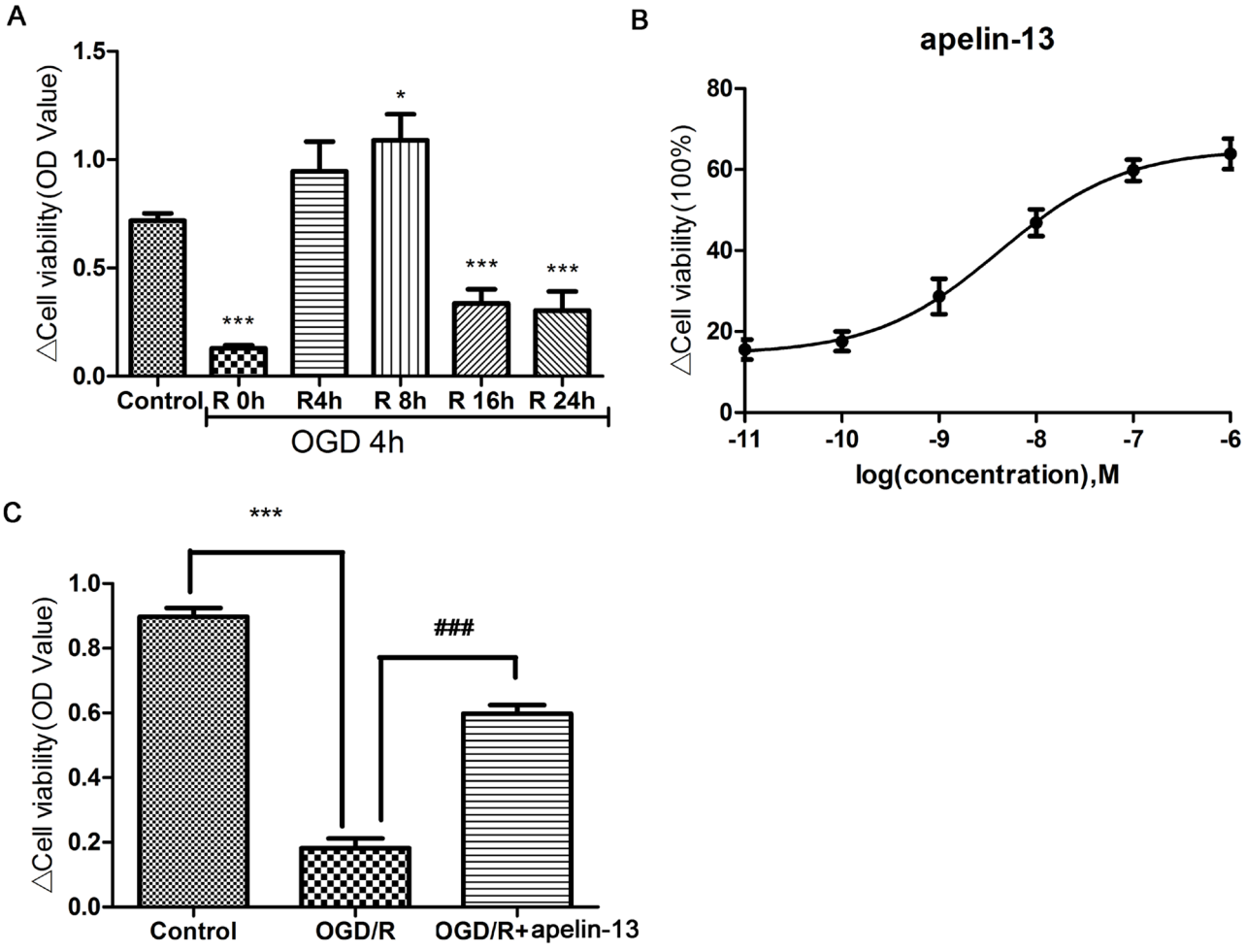

Figure 1. Oxygen–glucose deprivation/reoxygenation (OGD/R) decreases cell viability, and apelin-13 alleviates OGD/R injury and increases cell viability. (A) OGD4/R0 decreased cell viability and transiently increased cell viability following reoxygenation; however, 16 h after reoxygenation, cell viability decreased again; $^{***}P < 0.05$ versus control. (B) bEnd.3 cells were pretreated with various apelin-13 concentrations prior to OGD4/R16. The EC50 of apelin-13 was determined to be 4.171×10^{-9} M. (C) OGD4/R16 decreased cell viability ($^{***}P < 0.0001$ versus control), whereas apelin-13 (1×10^{-8} M) attenuated this decrease in viability ($^{###}P < 0.0001$ versus OGD4 h/R16 h). Data are shown as means ± SD ($n = 6$).

downregulated, indicating that OGD/R stress induced an increase in autophagy activity in endothelial cells and that apelin-13 further increased this induction. To confirm this result, we used immunofluorescence to detect expression and aggregation of LC3 II (Figure 3(E)). The level of LC3 II was much higher in the OGD/R group than in the control, and this effect was amplified in the OGD/R + apelin-13 group. The results of immunofluorescence analyses were consistent with those obtained by Western blotting.

Inhibition of autophagy exacerbates OGD/R injury and abolishes the protective effect of apelin-13 on TJ proteins

To further understand the role of autophagy in OGD/R-induced dysfunction in bEnd.3 cells, we applied 3-methyladenine (3-MA), a class III PI3K inhibitor, to block autophagy in the early stages of autophagosome formation. As shown in Figure 4(A) to (C), pretreatment with 3-MA (5 mM) for 30 min exacerbated OGD/R-induced damage to the TJ proteins occludin and ZO-1. Moreover, in OGD/R + apelin-13 samples pretreated with 3-MA, the protective effect of apelin-13 on TJ proteins was abolished. These data suggested that the protective effect of apelin-13 against OGD/R stress is mediated by induction of autophagy.

Involvement of autophagy in cell migration induced by apelin-13

To detect the effect of autophagy on cell migration, we measured the healing rate of endothelial cell scratches before reoxygenation (R0 h), and reoxygenation for 4, 16, 24, or 36 h after OGD. As shown in Figure 5(A) and (B), the healing rate did not differ significantly between groups at OGD4/R0, OGD4/R4, and OGD4/R36. At OGD4/R16 and OGD4/R24, pretreatment with 3-MA decreased the healing rate relative to the OGD/R groups without 3-MA, but the differences were not statistically significant. In the OGD/R + apelin-13 groups, pretreatment with 3-MA significantly decreased the healing rate. These results indicated that apelin-13 increased the migration capacity of endothelial cells by upregulating the autophagic activity induced by OGD/R; however, OGD/R-induced autophagic activity alone had no effect or was insufficient to influence migration.

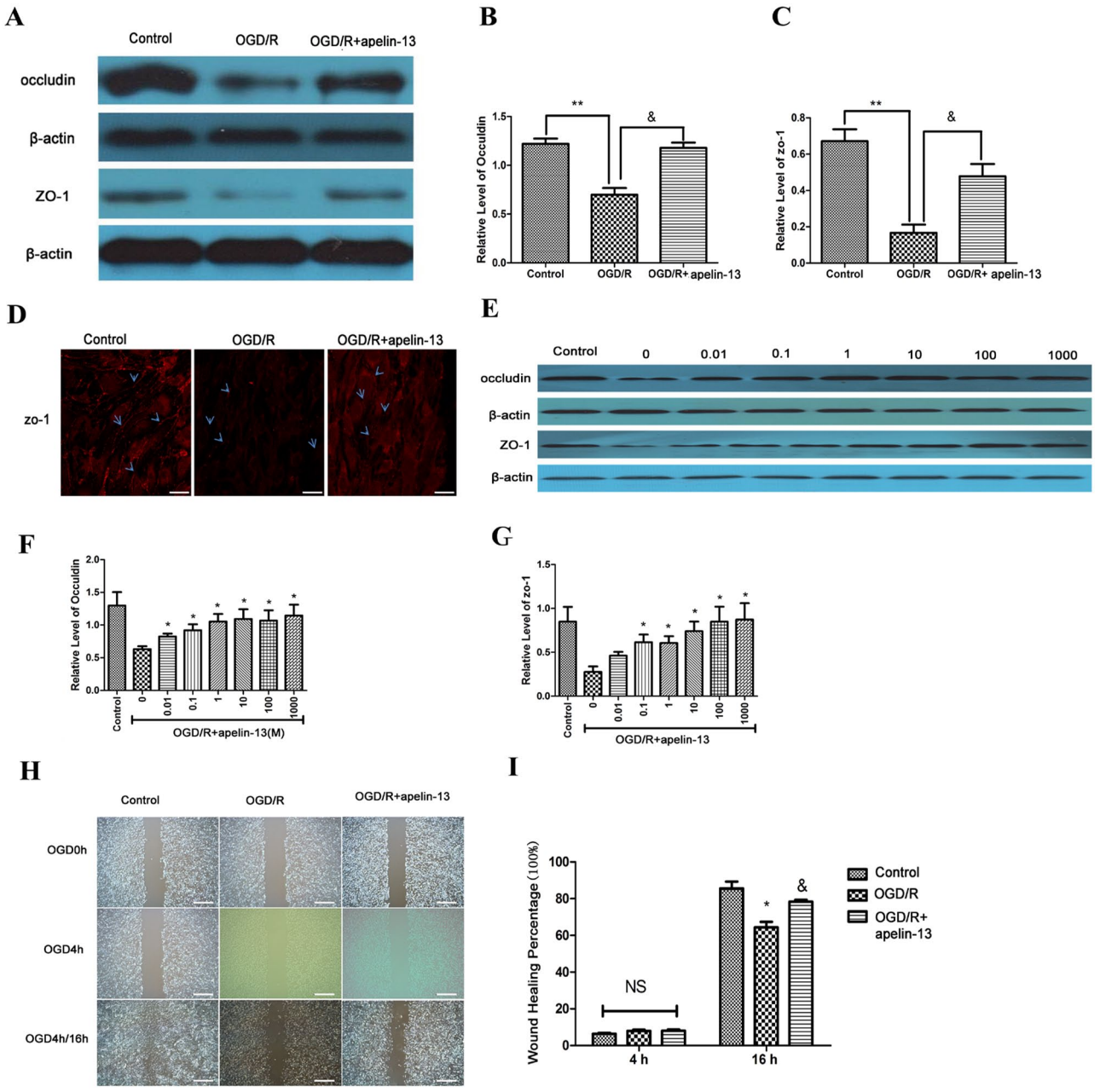

Figure 2. Apelin-13 alleviates the effects of OGD/R on TJs and cell migration. (A) Representative Western blot analysis of occludin and ZO-1. Densitometric analysis was used to quantify the levels of occludin (B) and ZO-1 (C). Values were normalized against β-actin. Data are shown as means ± SD, $n = 3$. $^{**}P < 0.05$ versus control; $^{\&\&}P < 0.05$ versus OGD4/R16. (D) Representative images showing the effect of OGD4/R16 and apelin-13 on the expression of ZO-1. Scale bars represent 25 μm. (E to G) Representative Western blot analysis of occludin and ZO-1 (E) and densitometric analyses demonstrating that protection of occludin (F) and ZO-1 (G) is dependent on the concentration of apelin-13. $^{*}P < 0.05$ versus OGD/R0. (H) Representative images showing that OGD/R-induced cell migration injury in monolayer-cultured bEnd.3 cells was abolished by pretreatment with apelin-13. Scale bars represent 100 μm (I) Quantification of the healing rate from three independent experiments. $^{*}P < 0.05$ versus control; $^{\&}P < 0.05$ versus OGD4/R16.

Apelin-13 promotes OGD/R-induced activation of autophagy by inhibiting the AKT/mTOR signaling pathway

Finally, we sought to identify the signaling pathway responsible for induction of autophagy by OGD/R and apelin-13. Because the AKT–mTOR signaling pathway plays important roles in regulating autophagy, we examined phosphorylation of AKT and mTOR. Relative to the control group, OGD/R significantly decreased the levels of phosphorylated AKT and mTOR, and apelin-13 treatment drove a further decrease in both levels. These results suggest that OGD/R and apelin-13 induce autophagy by inhibiting the AKT–mTOR signaling pathway (Figure 6).

Discussion

Apelin-13 has a protective effect on brain I/R injury. Apelin-13 effectively ameliorates neurological defects, decreases brain infarct volume, and reduces brain edema; in addition, it significantly attenuates apoptosis by downregulating Bax, caspase-3, and cleaved caspase-3 and upregulating Bcl-2.[28]

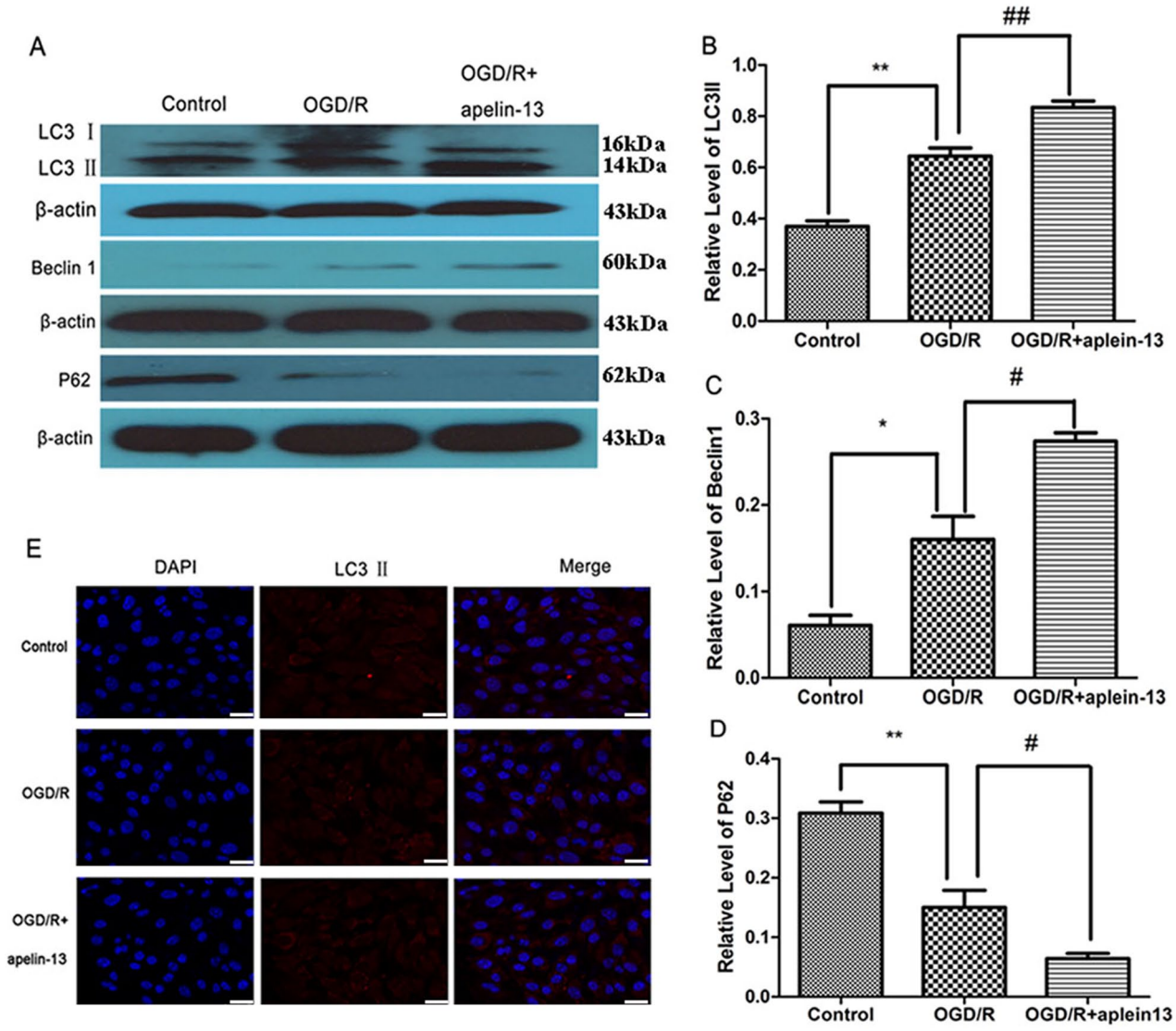

Figure 3. Apelin-13 promotes OGD/R injury–induced autophagy in bEnd.3 cells. (A) Western blot analysis of autophagy-related marker proteins LC3 II, beclin 1, and p62. OGD4/R16 upregulated endogenous LC3 II and beclin 1 and promoted degradation of p62; apelin-13 increased all of these effects. Bar charts show quantification of endogenous LC3 II (B), beclin 1 (C), and p62 (D). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus OGD4 h/R16 h. (E) Representative confocal images showing that LC3 II puncta in the OGD/R group accumulated to a greater extent in the apelin-13–treated group. Scale bars represent 25 μm.

Previously, we showed that intracerebroventricular (ICV) injection of apelin-13 decreases the volume of cerebral infarction and ameliorates inflammation in rats.[30,32] Disruption of the BBB, together with the subsequent brain edema, neuroinflammation, and free radical–mediated damage, contributes to the development of many neurodegenerative disorders, including Alzheimer's, Parkinson's, and Huntington's, and is associated with poor clinical outcomes. Most studies on the protective effect of apelin-13 against cerebral I/R injury have focused on the nervous system.[33–36] In this study, we used an OGD/R model, a classical model of cerebral ischemia, to test the protective effect of apelin-13 on TJs in bEnd.3 cells treated with OGD/R. Our goal was to characterize the ability of apelin-13 to attenuate disruption of the BBB following cerebral I/R injury.

Mounting evidence suggests that elevated BBB permeability is an important factor in the initiation of cerebrovascular disease and diabetic stroke.[37] The physiological function of Cerebral microvascular endothelial cells may be impaired during I/R. Accordingly, in this study, we employed bEnd.3 as an *in vitro* BBB model to investigate the role of apelin-13. The core structures of the BBB consist of TJs, which are in turn composed of transmembrane proteins and many membrane-related cytoplasmic proteins.[38] Occludin and ZO-1 play key roles in regulating the integrity and proper functions of the BBB. Cerebral I/R can cause the destruction and redistribution of both proteins, damaging the integrity of the BBB and increasing its permeability.[39,40] Our data showed that OGD/R decreased the expression levels of occludin and ZO-1 and disrupted the continuity of TJ protein staining, resulting in significant gaps; these observations are consistent with previous reports. Most important of all, our data showed that apelin-13 can attenuate the disruption of TJ proteins following OGD/R injury in bEnd.3 cells. Chu *et al.*[41]

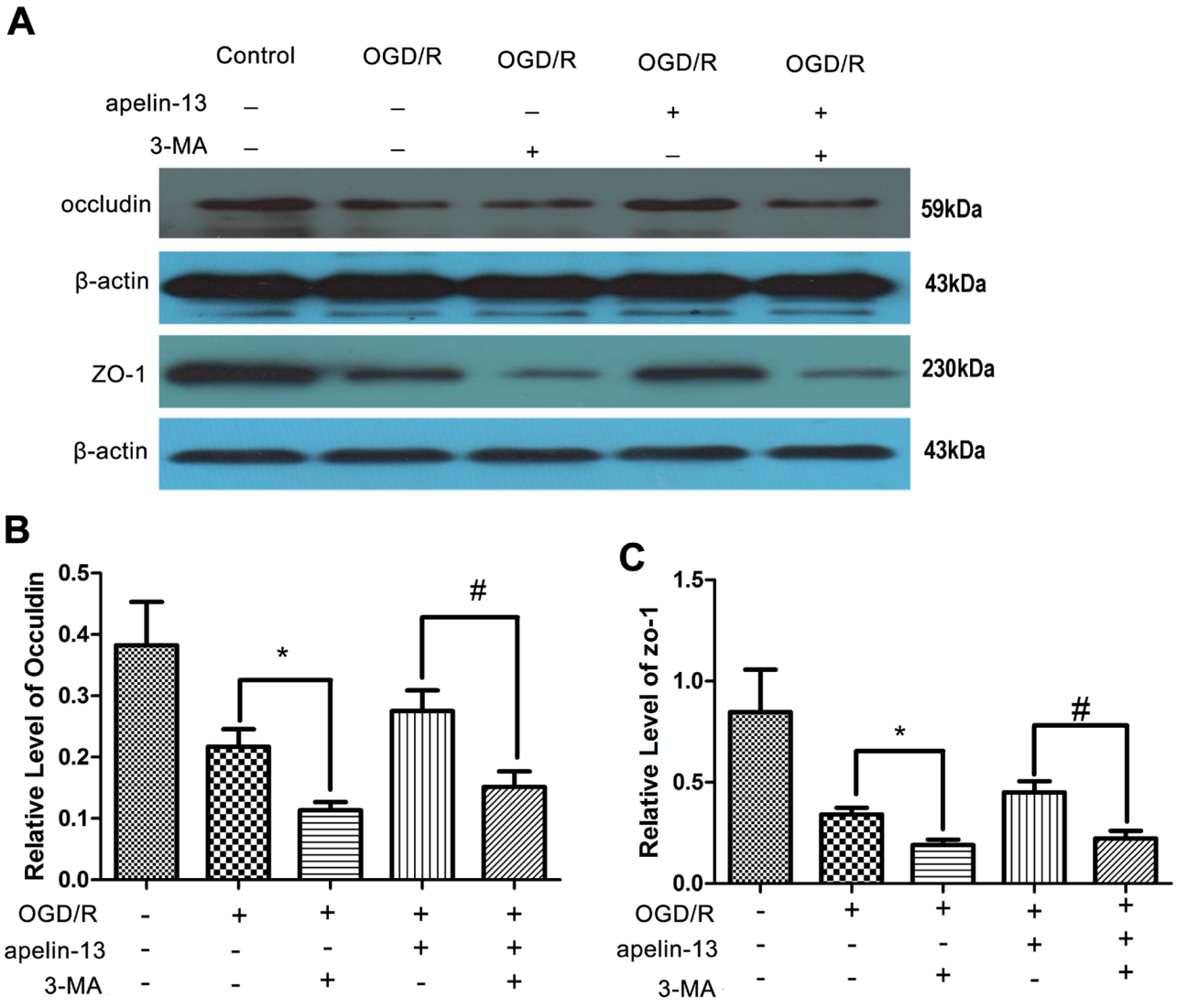

Figure 4. Involvement of autophagy in bEnd.3 cells in OGD/R injury and the protective effect of apelin-13. (A) Representative Western blots showing the effect of the autophagy-specific inhibitor 3-MA on ZO-1 and occludin levels. Densitometric analyses of ZO-1 (B) and occludin (C) from three independent experiments. $^{*}P < 0.05$ versus OGD4/R16; $^{\#}P < 0.05$ versus OGD4/R16 + apelin-13.

reported that apelin-13 protects multiple components of BBB; by monitoring morphology and function with electron microscopy, they showed that apelin-13 suppresses widening of the perivascular space widening, opening of TJs, swelling of capillary endothelial cells, and elevated BBB permeability to macromolecules following middle cerebral artery occlusion (MCAO). At the molecular level, our study shows that apelin-13 not only protects cerebral microvascular endothelial cells from damage caused by anoxia and lack of glucose, but it also enables cells to resist the inevitable secondary damage caused by the recovery of oxygen and sugar supply.

Once autophagy is initiated, microtubule-associated protein 1 LC3 (light chain 3) in the cytosol is cleaved to soluble LC3 I, which is then converted to the lipid-bound form LC3 II. This process is associated with the formation of autophagosomes. One of the key regulators of autophagosome formation by membrane recruitment is beclin 1; accordingly, in addition to accumulation of LC3 II, beclin 1 was also chosen as a hallmark of upregulation of autophagy. We observed stimulation of autophagy (reflected by elevated levels of LC3 II and beclin 1) in our OGD/R cell model, in good agreement with the previous reports.[42,43] We also observed formation and accumulation of LC3 puncta. During the physiological process of autophagosome maturation, p62 is delivered to autophagosomes, leading to the degradation of trapped substrates (including p62) by lysosomal enzymes; this phenomenon is referred to as autophagic flux.[42,44] The increase in the levels of LC3 II and beclin 1 might be due to bona fide induction of autophagy, but might also be due to accumulation of autophagosomes caused by the disrupted autophagic flux. Therefore, in our experiments, we chose the p62 as a negative marker of autophagic induction, along with LC3 II and beclin 1. Our data revealed that p62 levels significantly decreased in OGD/R-treated bEnd.3 cells, in good agreement with previous reports. Notably in this regard, apelin-13 further increased OGD/R-induced autophagic flux, reflected by upregulation of LC3 II and beclin 1 and degradation of p62.

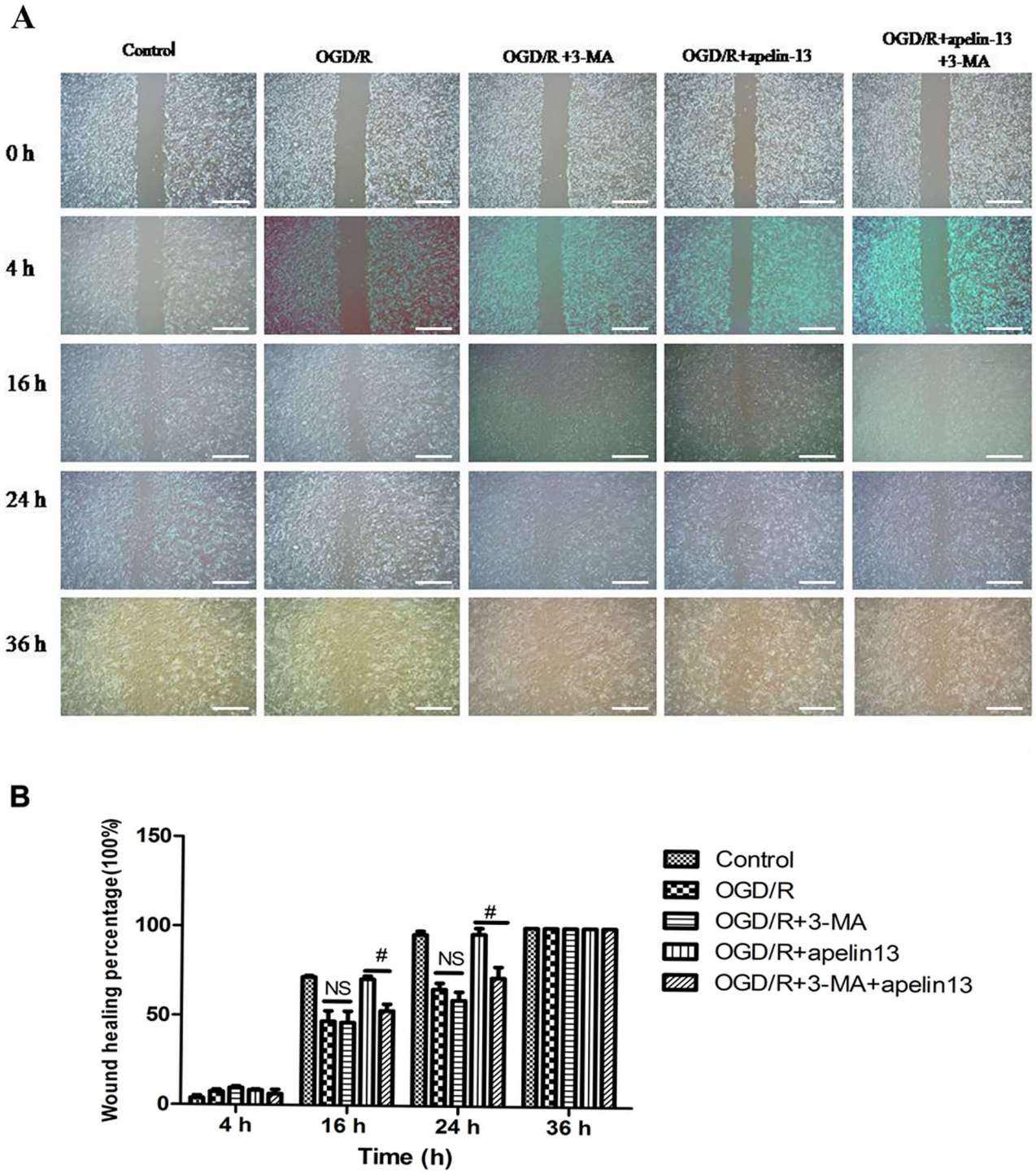

Figure 5. Involvement of autophagy in the protective effect of apelin-13 on migration in OGD/R-injured bEnd.3 cells. (A) Representative images depicting the effect of 3-MA on cell migration. (B) Quantification of the healing rate from three independent experiments. In OGD4/R16: NS $P = 0.9455$ versus OGD/R; $^{\#}P = 0.0136$ versus OGD/R + apelin-13. In OGD4/R24: NS $P = 0.3525$ versus OGD/R; $^{\#}P = 0.0274$ versus OGD/R + apelin-13. Scale bars represent 100 μm.

Autophagy has long been seen as a double-edged sword: an appropriate physiological level of autophagy is protective and helps to maintain basal homeostasis, whereas excessive autophagy can be detrimental to BBB integrity.[45] Zeng *et al*.[46] reported that I/R injury induces p65/beclin 1–dependent autophagy, leading to autophagic death, in human umbilical vein endothelial cells (HUVECs). Similarly, excessive activation of autophagy in obese/hypertensive rats causes endothelial cell dysfunction.[47] However, recent studies showed that autophagy in cerebral microvascular endothelial cells is activated in diabetic rats with permanent occlusion of middle cerebral artery (pMCAO), and that chloroquine, an autophagy inhibitor, aggravates pMCAO-induced BBB leakage.[48] In addition, upregulation of autophagy in brain

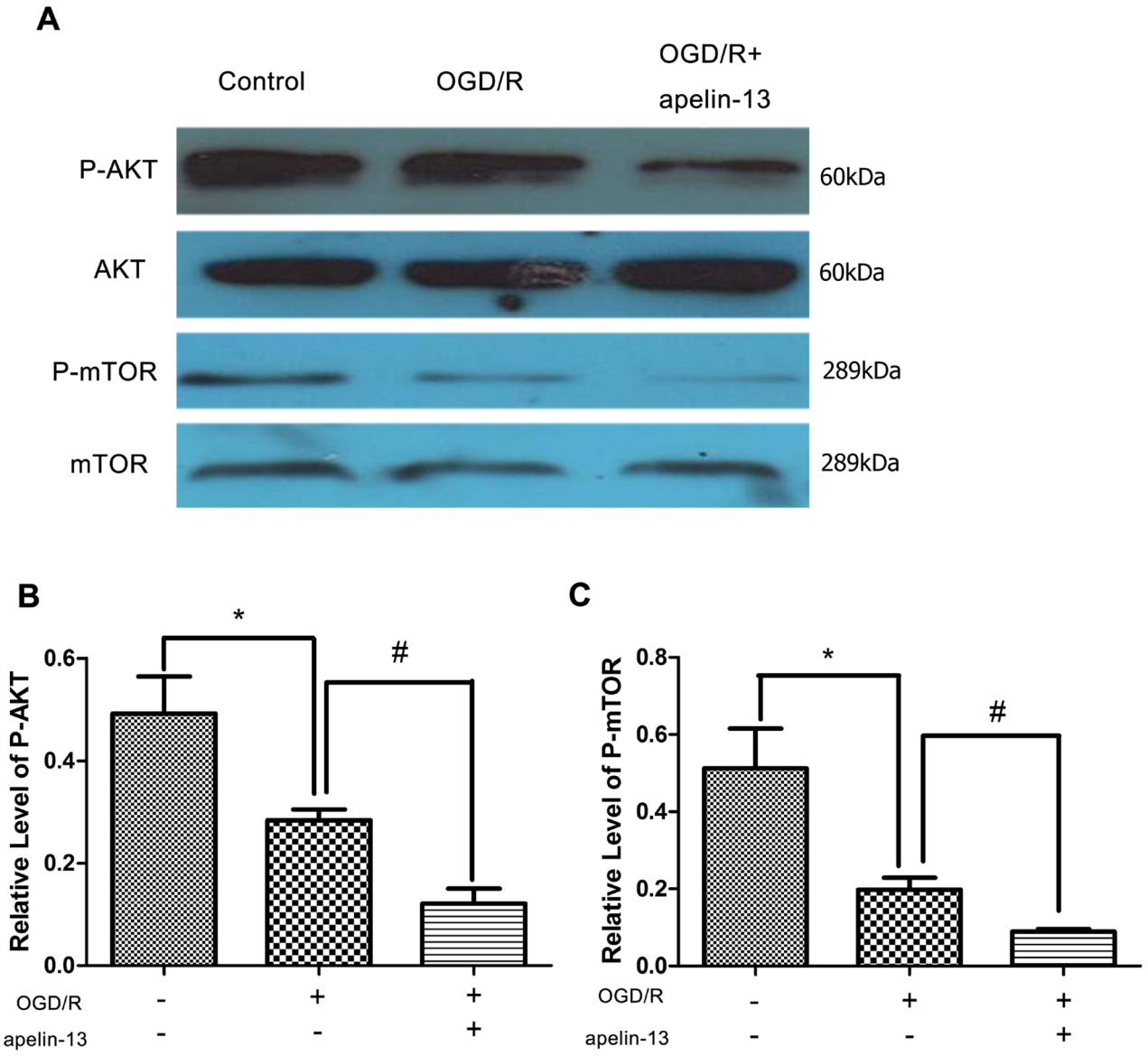

Figure 6. OGD/R and apelin-13 inhibit the AKT–mTOR signaling pathway. (A) Western blots for AKT, mTOR, and the corresponding phosphoproteins. Densitometric analysis was used to quantify the phosphorylation levels of AKT (B) and mTOR (C). Values are expressed as means ± SD ($n = 3$). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus OGD/R.

microvascular endothelial cells (BMVECs) during I/R has a potential protective effect on BBB integrity.[49] Our data showed that the reduction in TJ protein expression was related to the increase in OGD/R-induced autophagy. However, treatment with the autophagy-specific inhibitor 3-MA further decreased expression of ZO-1 and occludin, indicating that the increase in OGD/R-induced autophagy in bEnd.3 cells can alleviate degradation of TJ proteins caused by OGD/R injury. Apelin-13 upregulated the expression of TJ proteins in bEnd.3 cells treated with OGD/R, but after pretreatment with 3-MA, the protective effect of apelin-13 was abolished. This implies that apelin-13 protects cerebral microvascular endothelial cells from OGD/R injury by boosting autophagic activity. Mounting evidence supports the idea that apelin-13 attenuates BBB injury induced by I/R damage. Apelin-13 protects the BBB from disruption by cerebral ischemia both morphologically and functionally,[41] and also protects the BBB from hemorrhagic damage.[50] Our data showed that apelin-13 protects TJ proteins from disruption caused by OGD/R injury by enhancing OGD/R-induced autophagic activity. Thus, autophagy represents new targets for drug development and treatment of various diseases including ischemic stroke. Moreover, although reperfusion can interfere with cellular homeostasis, upregulation of autophagy can alleviate reperfusion injury, making cells better able to survive.

Both thrombosis and vascular recanalization involve damage to vascular endothelial cells. Endothelial cell migration plays important roles in angiogenesis, wound healing, and endothelial injury healing.[45,51,52] Therefore, we studied the effects of OGD/R and apelin-13 on endothelial cell migration. In the early stage of reperfusion (R16 h and R24 h), OGD/R decreased the migration capacity of cells, suggesting that reperfusion injury prevents migration by endothelial

cells at the early stage. However, the inhibition of autophagy by 3-MA in the OGD/R group did not alleviate the loss of migration capacity, that is, OGD/R-induced autophagy could not or was insufficient to alleviate the loss of migration capacity caused by OGD/R. However, the autophagy induced by apelin-13 increased the migration capacity of vascular endothelium cells. In the late stage of reperfusion (R36 h), apelin-13 did not affect the migration capacity of endothelial cells, probably because the vascular endothelium had returned to normal by this time. Similarly, apelin-13 had no effect on proliferation and angiogenesis in normal vascular endothelial cells.

Apelin-13 may protect the BBB from damage after cerebral ischemia by activating ERK and PI3K/AKT pathways in astrocytes.[41] In addition, Yang *et al*.[31] showed that ICV injection of apelin-13 protects the mouse brain against I/R injury by activating the PI3K/AKT and ERK1/2 signaling pathways. However, our data showed that OGD/R can downregulate the AKT/mTOR pathway in cultured bEnd.3 cells, and that apelin-13 treatment can strengthen this effect. Multiple signaling molecules, including MAPKs, mTOR, and class III PI3Ks, have been implicated in regulation of autophagy.[53,54] The PI3K/AKT pathway can negatively regulate autophagy via phosphorylation of mTOR, potentially explaining why we observed downregulation of AKT and mTOR, leading to an increase in autophagic activity. Our results showed that OGD/R and apelin-13 could inhibit AKT/mTOR, thereby upregulating autophagy activity and protecting bEnd.3 cells from OGD/R injury.

Conclusions

We have shown that OGD/R stress can induce autophagic activity in cerebral microvascular endothelial cells. In addition, we found that apelin-13 protects TJ proteins in adjacent cells and cellular migration capacity by upregulating autophagic activity induced by OGD/R injury. These observations support the idea that apelin-13 could be used to develop a novel therapeutic approach to I/R injury.

AUTHORS' CONTRIBUTIONS

Conceptualization and methodology: JC and BB; data curation and writing – original draft preparation: RZ; writing – review and editing: JC, FW, and RZ; supervision: BC; project administration: CW; funding acquisition: BB, RZ, FW, and CW. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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