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Original Article

Role of autophagy and its signaling pathways in ischemia/reperfusion injury

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Abstract: This study was conducted to investigate the mechanism of autophagy and its signaling pathways in ischemia/reperfusion injury (IRI). Pulmonary microvascular endothelial cells (PMVECs) were used to construct I/R models. The cells were then treated with autophagy inhibitor 3-MA and infected with adenovirus expressing Beclin 1-shRNA. The expression of CD31, LC3-II, Bcl-2, Bax, LC3-II, Beclin 1, AKT, p-AKT, AMPK and p-AMPK, apoptosis, cell viability and migration ability were determined. Over 95% isolated PMVECs were positive for CD31. The expression of LC3-II and Beclin 1 was up-regulated in I/R cells. 3-MA and Beclin 1 knockdown inhibited the expression of LC3-II and Beclin 1 and autophagosome formation. Autophagy induced by hypoxia was antagonistic against apoptosis, which increased after treatment with 3-MA and knockdown of Beclin 1. 3-MA and Beclin 1 knockdown downregulated and upregulated the expression of Bcl-2 and Bax, respectively. Apoptosis mediated by hypoxia and reperfusion-induced autophagy was reduced by 3-MA and Beclin-1 knockdown, which increased and reduced the expression of Bcl-2 and Bax, respectively, leading to significant decreased Bax/Bcl-2 ratio. In these cells, expression of p-AKT, p-AMPK and p-mTOR was up-regulated. After treatment with 3-MA and Beclin 1 knockdown, expression of p-AKT and p-AMPK was significantly reduced.

Keywords: Autophagy, signaling pathways, ischemia/reperfusion injury, apoptosis

Introduction

Primary graft dysfunction (PGD) induced by ischemia reperfusion (I/R) injury (IRI) is one of the main causes of perioperative death in lung transplantation [1]. IRI in donor lung may generate reactive oxygen species (ROS), calcium overload, release and activation of inflammatory cytokines and alexins, destruction of blood coagulation and fibrinolysis balance, and massive infiltration of inflammatory cells, eventually leading to dysfunction or death of pulmonary microvascular endothelial cells, and subsequently reduced permeability of the pulmonary vascular vessels and damaged pulmonary diffusion function [2]. Current interventions for lung transplantation IRI mainly focus on inhibiting inflammatory response to alleviate IRI in donor lung. More and more studies have shown that cell death is also an important pathological change in IRI after lung transplantation. When donor lung had cold ischemia for more than 6 hours, large number of cells were dead [3, 4] . Inhibition of caspase-8 a key enzyme in apoptosis pathway, is shown to reduce apoptosis after lung transplantation and improve the function of donor lung after transplantation [5]. In vitro experiments have shown that HR reduces the viability of cultured lung cells and induce cell death. However, little has been done to alleviate IRI after lung transplantation by regulating cell death and intracellular homeostasis.

Autophagy is a well-studied cell homeostasis, in which autophagosomes are formed by fusing injured large molecules such as proteins and injured organelles with lysosomes. In the process, the injured proteins and organelles are enzymatically degenerated to produce energy and substrates for re-synthesis of organelles to maintain cell functions [6, 7]. Therefore, autophagy is important for cell survival, differentiation and homeostasis [8]. Meanwhile, autophagy also induces cell death. Excessive autophagy disrupts proteins and organelles, leading to irreversible cell atrophy and death (autophagic

cell death). In addition, autophagy may induce apoptosis and necrosis. In the dying cells, autophagy is often highly expressed and is activated for signal transduction pathways related to apoptosis [9, 10].

In previous studies, we found that autophagy is involved in IRI in lung transplantation [11]. However, the role of autophagy in the apoptosis of pulmonary microvascular endothelial cells (PMVECs) has not been reported. In this study, we further investigated the role and mechanism of autophagy in the apoptosis of microvascular endothelial cells using isolated PMVEC IR models. The findings would provide new insights into the mechanism of IRI-mediated graft injury in lung transplantation and improve the success rate of transplantation.

Materials and methods

Animals

Healthy male SD rats, weighing 120 to 150 g and aged 2-3 months, were purchased from the Experimental Animal Center, Nanchang University. All animal experimental protocols were approved by School of Medicine, Nanchang University. All animals received humane care in compliance with the 'Principles of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996).

Isolation and culture of PMVECs

PMVECs were isolated as previously reported [12]. Briefly, rats were anesthetized and the thoracic cavity was cut open to drain. Lung tissue under the pleura was isolated and tissue at 1 mm depth of the lung edge was cut into 1-3 mm^3 pieces, immersed and cultured in DMEM complete medium with fetal bovine serum (Gibco, USA). The medium was refreshed every day to remove blood cells. After adhered to the walls for 60 h, the tissues were gently removed and the cells were cultured till reaching a confluency of 90%. The cells were then digested with 0.25% trypsin and passaged. The cells at the third passage were examined for presence of endothelial marker CD31 using immunofluorescence assay before being used for the experiments.

Hypoxia- reperfusion and drug treatment

The cells were subjected to normal culture (Nor), hypoxia culture (Hypoxia) and hypoxia-reperfusion (H/R) with (3-MA, 10 nM, Sigma, USA) and without 3-MA (Control). Each treatment was conducted in three wells (three repeats). For hypoxia group, cells were cultured in hypoxia incubator (50% N_2, 5% CO_2, 45% air) for 8 h. For H/R group, cells were cultured in regular incubator (5% CO_2, 95% air) after being cultured in hypoxia incubator for 6 h. All experiments were repeated three times independently.

Cell viability assay

PMVECs were inoculated to the wells of 96 well plates (100 μL/well), cultured for 24 h at 37°C in 5% CO_2, and added with 10 μL CCK solution ((Sigma-Aldrich, US). After additional 1-4 h culture, the absorbance at 450 nm was measured with a microplate reader.

Flow cytometry

100 μl PMVECs in early logarithmic growth phase was seeded in the wells of 6 well plates (1×10^5 cells/ml), cultured for 48 h and harvested by centrifugation at 800 rpm for 5 min. The cells were re-suspended in 50 μl PBS and added with 500 μl binding buffer, 5 μl AnnexinV-FITC and 5 μl propidium iodide. The mixtures were incubated in the dark at room temperature for 10 m, washed three time with PBS and loaded to a flow cytometer (FACSCalibur, BD, USA) for apoptosis analysis.

Immunofluorescence assay

PMVECs in logarithmic growth phase were seeded in the slides in 12-well plates (1×10^4 cells/slide) and treated as described above. The slides were rinsed three times with PBS and fixed in 4% paraformaldehyde for 15 min. After rinsed with PBS, the slides were blocked with PBS containing 1% goat serum for 30 m at room temperature, incubated with diluted CD31 or LC3-II antibodies (Abcam, USA) overnight and then with rhodamine-labeled goat anti-rabbit secondary antibody (Abcam, USA) in the darkness at room temperature for 1 h. The

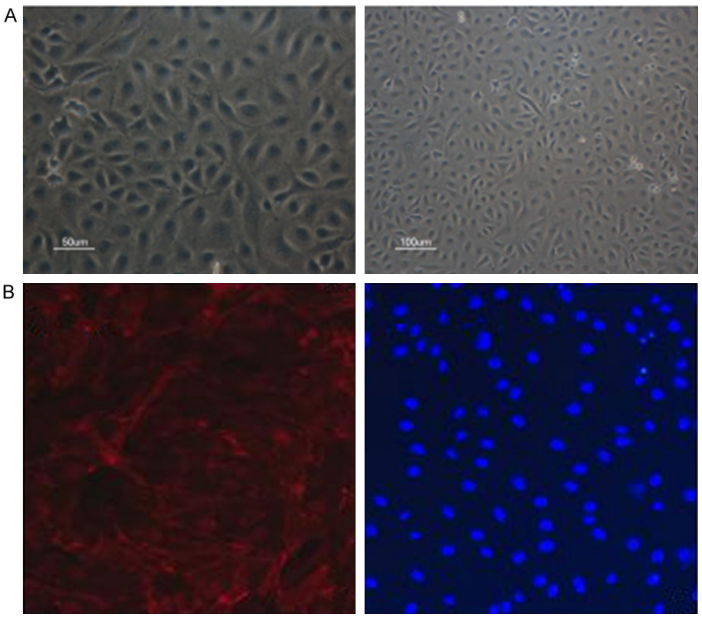

Figure 1. Morphology and CD31 immunostaining of PMVECs at the third passage. A. microphotos at 400 × and 200 ×, bar indicates 50 μm. B. Immunostaining of CD31 (1000 ×, bar indicates 2 μm).

slides were stained with DAPI for 5 m, sealed with solution containing anti-fluorescence quenching agent and viewed and pictured with a fluorescence microscope.

Cell migration assay

The treated cells were cultured for 48 h, digested and added to the upper wells of Transwell chambers (Corning, USA). After 24 h, the inserts were fixed in paraformaldehyde, stained with crystal violet and the number of migrated cells in 5 fields were counted under an inverted microscope.

Western blot analysis

The treated cells in logarithmic growth phase were harvested and lysed in 400 μl NP-40 lysis buffer. The total proteins in the supernatants were determined using the BCA kit and 20 μg proteins were separated by SDS-PAGE gel electrophoresis, transferred to membranes, and staining with antibodies against Bcl-2, Bax, LC3-II, Beclin 1, AMPK, p-AMPK, Akt, p-Akt, P-JNK, JNK and GAPDH (loading control) (Abcam, USA) and goat anti- rat horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, USA). Immunoreactive bands were visualized using Western Lighting Chemiluminescence Reagent Plus (PerkinElmer, USA) according to the manufacturer's instructions, and then quantified by densitometry using a ChemiGenius Gel Bio Imaging System (Syngene, USA).

Adenovirus expression vector and transfection

Rat Beclin 1-shRNA adenovirus and negative control were obtained from Hanheng Biological, Beijing, China. Three sequences for Beclin 1 siRNA were designed (#1, 5'-CACCTATCCACGAGCTCACTGCTCCGTTACGAATAACGGAGAGTGAGCTCGTGGATAAAAATATCCACGAGCTCACTGCTCCGTTATTCGTAACGGAGAGTGAGCTCGTGGATA, #2, 5'-CACCCACACTTATTGGAGAAGCTTCTGATCGAAATCAGAAGCTTCTCCAATAAGTGTGAAAACACACTTATTGGAGAAGCTTCTGATTTCGATCAGAAGCTTCTCCAATAAGTGTG, #3, 5'-CACCTGGAGAACTTGAGTCGCAGACTGAACGAATTCAGTCTGCGACTCAAGTTCTCCAAAAATGGAGAACTTGAGTCGCAGACTGAATTCGTTCAGTCTGCGACTCAAGTTCTCCA) to construct adenovirus expression vector based on pBHGlox (delta) E1, 3Cre to transfect cells. Negative control (Scramble) sequence was 5,-CGAGTGAGCTCGTGGATAACGAGCTCACTGCTCCGTTACGAATAACGGAACCTATCC. PMVECs were cultured in DMEM medium containing 10% FBS at 37°C in 5% CO_2 for 24 h and transfected with the virus and negative control. The transfected cells were cultured in different oxygen conditions as described above for 24 h and the medium was refreshed. 72 h later, the cells were assayed for viability, migration ability, apoptosis and expression of relevant proteins.

Statistical analysis

Data were expressed at means ± standard error and analyzed using SPSS16.0 statistical software. The Shapiro-Wilk test showed that the data were normality. The homogeneity of variance was verified by F test. Two factor analysis of variance was used for paired comparisons by the LSD-t test, $P < 0.05$ was considered statistically significant.

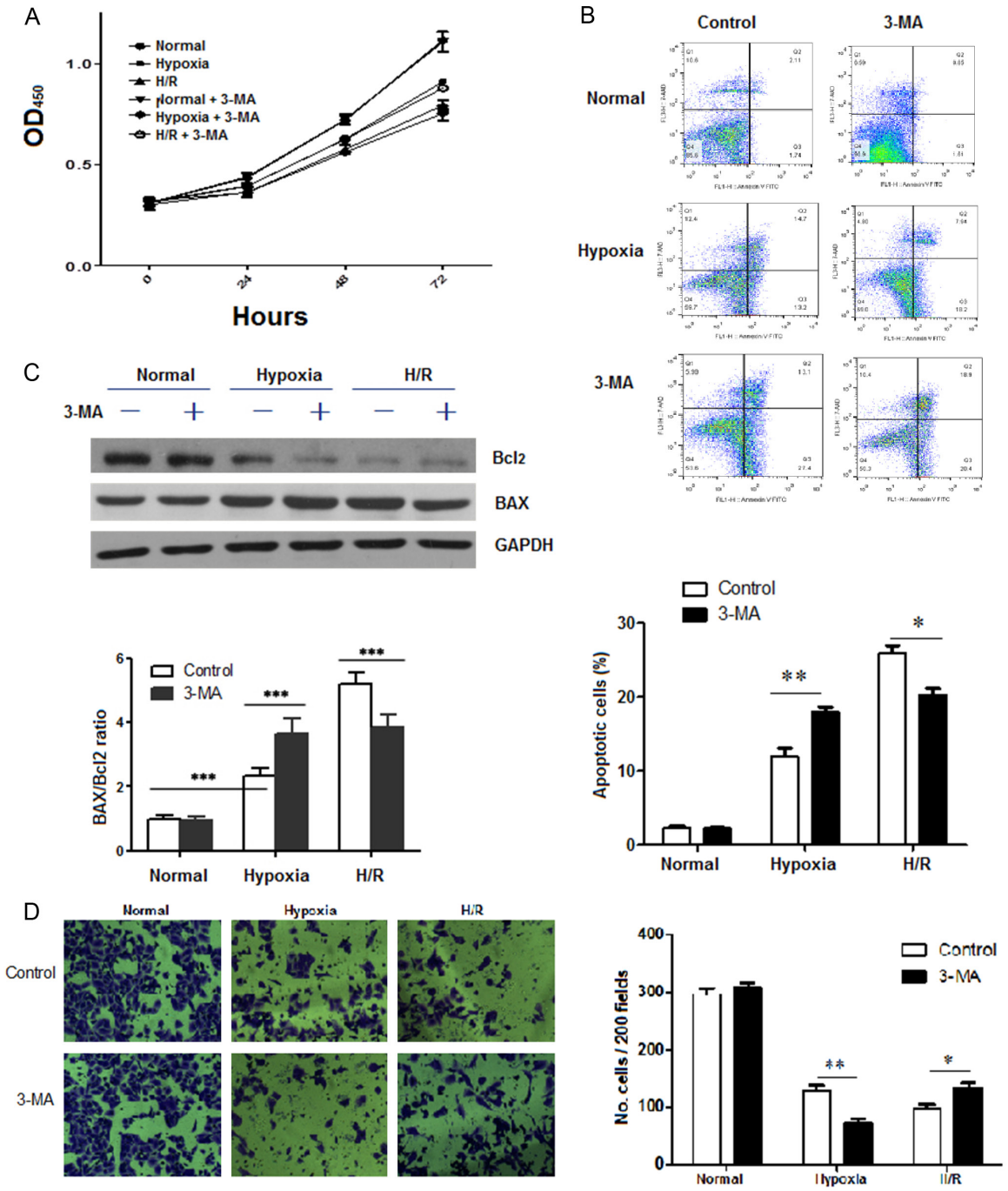

Figure 2. Viability, apoptosis, migration ability and expression of relevant proteins in PMVECs following hypoxia and H/R and 3-MA treatments. A. Cell viability assayed with CCK8 staining. B. Upper panel: representative flow cytometry results, lower panel: apoptotic rate. C. Upper panel: representative Western blots, lower panel: relative protein levels. D. Migration ability assayed using Transwell method. Left panel: microphotos showing migrated cells, right panel: number of migrated cells. *$P < 0.05$, **$P < 0.01 < 0.01$ between the groups under the bar.

Results

Characterization of PMVECs

PMVECs were isolated and cultured for three passages before being used for experiments. Microscopy showed that the cells were compactly arranged in typical cobblestone paving and were spindle- or polygon-shaped (**Figure 1A**). In addition, immunofluorescence assays showed that the over 95% of cells were positive for PMVEC marker CD31 (**Figure 1B**). These

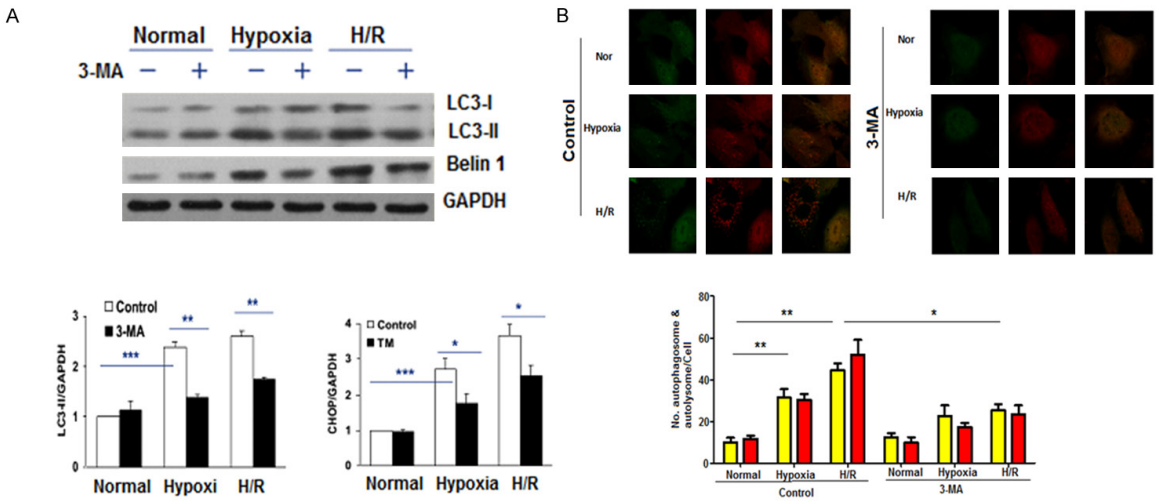

Figure 3. Expression of autophagy-related proteins and formation of autophagosome in hypoxia-, H/R- and 3-MA-treated PMVECs. A. Upper panel: representative Western blots, lower panel: Relative protein levels. B. Upper panel: microphotos of immunofluorescence, lower panel: number of autophagosome. *P < 0.05, **P < 0.01, ***P < 0.001 between the groups under the bar.

observations and the source of cells confirmed that the isolated cells were PMVECs.

Effects of autophagy on cell viability

PMVECs were subjected to various treatments and viability assays showed that hypoxia and H/R significantly inhibited the proliferation of the cells. Addition of 3-MA significantly reduced viability of hypoxia-stressed cells but increased the proliferation of H/R-treated cells (**Figure 2A**).

Effects of autophagy on cell apoptosis and relevant proteins

As shown in **Figure 2B** hypoxia effectively induced apoptosis in the cells, which was further increased after treatment with autophagy inhibitor 3-MA (P < 0.01), suggesting that during hypoxia autophagy plays a protective role. H/R treatment resulted in increased apoptosis and 3-MA intervention effectively inhibited the apoptosis (P < 0.01), suggesting that H/R treatment results in autophagy-mediated apoptosis in PMVECs.

Western blot analyses showed that after H/R treatment the expression of anti-apoptotic protein Bcl-2 was down-regulated and the expression of proapoptotic protein Bax was up-regulated, leading to significantly increased Bax/Bcl-2 ratio (P < 0.001, **Figure 2C**); 3-MA intervention further down-regulated Bcl-2 and up-regulated Bax, leading to greater Bax/Bcl-2 ratio (**Figure 2C**, P < 0.001). These results suggest that autophagy is antagonistic against apoptosis. After H/R treatment, the expression of Bcl-2 and Bax was decreased and increased, respectively, as compared with hypoxia group. However, 3-MA further increased the expression of Bcl-2 and decreased the expression of Bax, resulting in significantly reduced Bax/Bcl-2 ratio (**Figure 2C**, P < 0.001), suggesting that autophagy promotes apoptosis.

Effects of autophagy on cell migration ability

We then assayed the migration ability of the cells after hypoxia and H/R treatments. Hypoxia resulted in significant reduction in migration ability, which was further reduced following 3-MA treatment (**Figure 2D**, P < 0.01), suggesting that 3-MA significantly decreases the migration ability of the endothelial cells after ischemia. The migration ability of PMVECs was also decreased after H/R treatment but increased following 3-MA treatment (**Figure 2D**, P < 0.05), indicating that 3-MA enhances the reduced migration ability of endothelial cells due to H/R treatment.

H/R treatment enhanced autophagy in PMVECs

After hypoxia treatment, the expression of LC3-II and Beclin 1 was significantly up-regulated as compared with normal culture (**Figure 3A**, P <

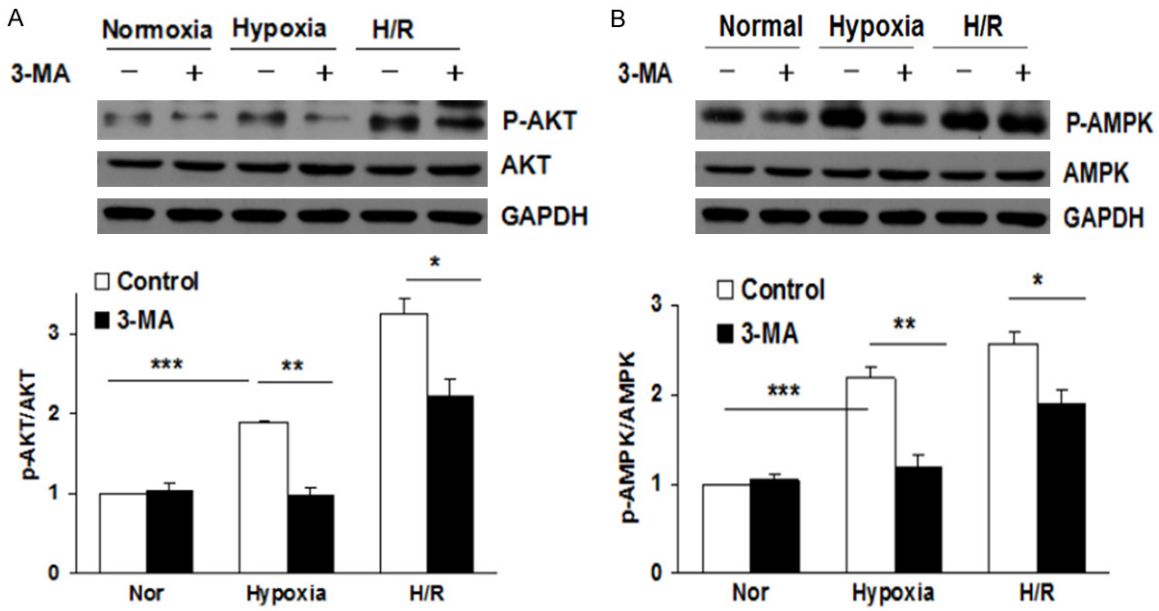

Figure 4. Expression of AKT, p-AKT, AMPK and p-AMPK in hypoxia-, H/R- and 3-MA-treated PMVECs. A. Upper panel: representative Western blots, lower panel: p-AKT/AKT ratio. B. Upper panel: representative Western blots, lower panel: p-AMPK/AMPK ratio. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 between the groups under the bar.

0.001). Reperfusion treatment further increased the expression of the two genes. Autophagy inhibitor 3-MA did not influence the expression of the two genes in normally cultured PMVECs, but significantly down-regulated the expression of the two genes after hypoxia and further after reperfusion treatment (**Figure 3A**, $P < 0.05$). To observe the changes in autophagosome production in PMVECs, we examined the expression of LC3-II after hypoxia and H/R treatment using immunofluorescence assays. Observations showed that in normally cultured PMVECs LC3-II was weakly expressed and the fluorescence was diffusing, while after hypoxia treatment, the expression of LC3-II was enhanced and the fluorescence appeared as round dots uniformly distributing on the surface of autophagosomes. The expression was further increased following reperfusion treatment. On other hand, 3-MA effectively reduced the fluorescence intensity and inhibited the formation of autophagosomes. The fluorescence from LC3-II appeared diffused after 3-MA treatment (**Figure 3B**).

Activated Akt-AMPK pathway induced autophagy

Next, we examined the activation of the Akt-AMPK pathway and its role in autophagy. It was found that after hypoxia treatment, the expression of p-AKT and p-AKT/AKT ratio were significantly increased in the cells (**Figure 4A**, $P < 0.001$); 3-MA treatment reduced the expression and ratio significantly (**Figure 4A**, $P < 0.001$). Furthermore, H/R treatment further increased the expression and the ratio (**Figure 4A**, $P < 0.01$), while 3-MA reduced the expression and the ratio (**Figure 4A**, $P < 0.05$). In addition, the expression of p-AMPK showed a similar pattern of change to p-AKT in response to oxygen and 3-MA treatments (**Figure 4B**). These findings suggest that the Akt-AMPK signaling may be involved in H/R-induced autophagy.

Knockdown of Beclin 1 by siRNA

To knockdown Beclin 1, three siRNAs were initially tested and Beclin 1-shRNA1 was found to be most effective (data not shown). The siRNA adenovirus was then used to infect PMVECs. It was found that once the autophagy-related gene Beclin 1 was knockdown, significant increase in apoptosis was observed in the cells following hypoxia treatment, which was, however, inhibited by H/R treatment (**Figure 5A**). Examination of the expression of apoptosis-related proteins showed that Beclin 1 knockdown resulted in increased BAX and decreased Bcl2 expression in PMVECs after hypoxia treat-

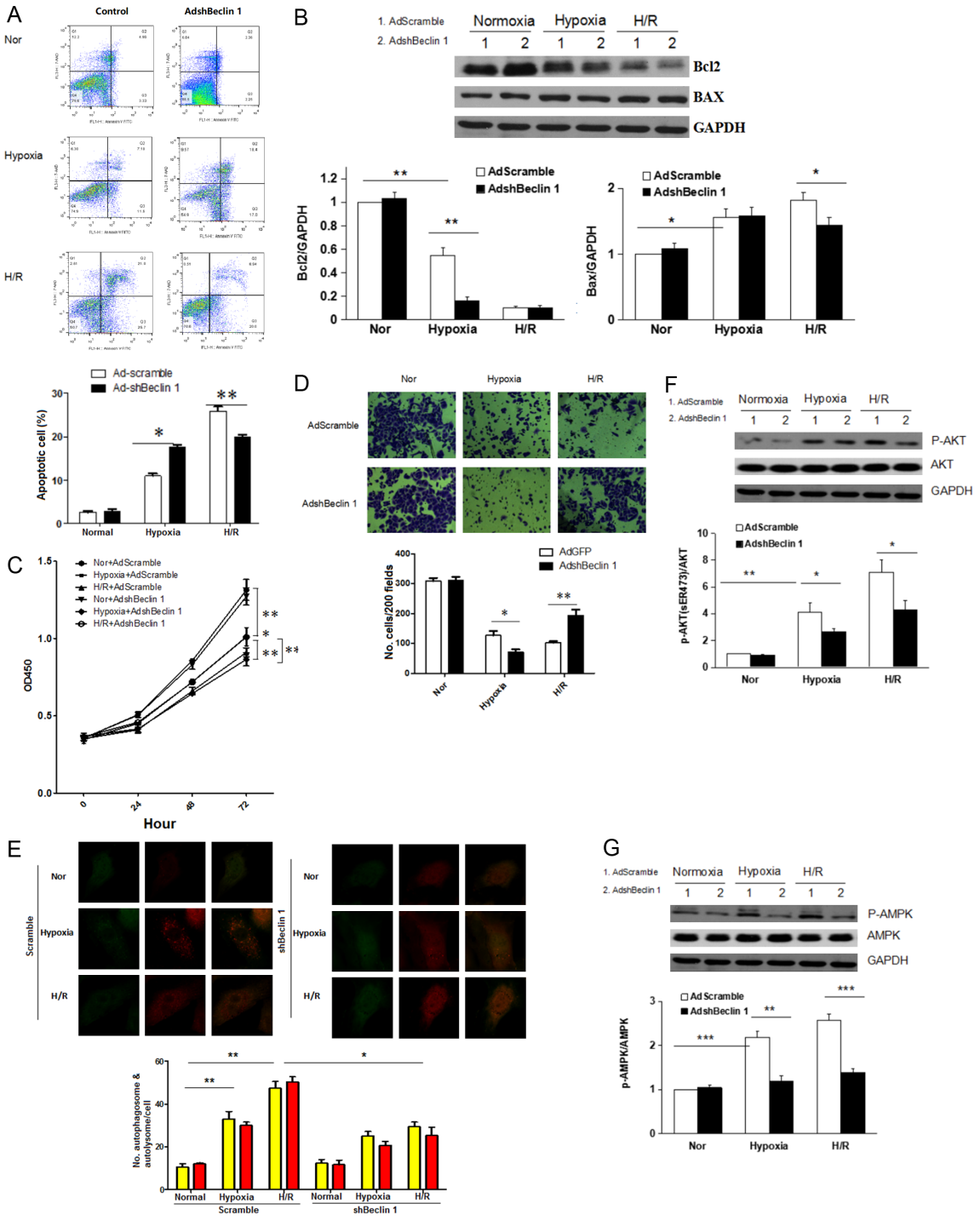

Figure 5. Apoptosis, expression of apoptosis and autophagy-related proteins, viability, migration ability of PMVECs following Beclin 1 knockdown. A. Upper panel: flow cytometry results, low panel: apoptotic rate; B. Upper panel: representative Western blots, low panel: relative expression of BAX and Bcl2; C: Viability of PMVECs; D. Upper panel: microphoto of migrated cells, low panel: number of migrated cells in Transwall assay; E. Upper panel: microphotos of autophagosomes and autolysomes, low panel: numbers of autophagosomes and autolysomes; F and G: Upper panel: representative Western blot, low panel: p-AKT/AKT and p-AMPK/AMPK ratio. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ between the groups under the bar.

ment, but decreased BAX after H/R treatment, although the expression of Bcl2 was not affected (**Figure 5B**). In addition, the viability of PMVECs was promoted or reduced after hypox-

ia or H/R treatment following the knockdown, respectively (**Figure 5C**). The migration ability of PMVECs was significantly reduced after Beclin 1 knockdown, but significantly increased following H/R treatment (**Figure 5D**). As shown in **Figure 5E**, autophagy was activated during hypoxia treatment and further after H/R, resulting in significantly increased numbers of autophagosomes and autolysosomes (**Figure 5E**). Downregulation of the Beclin 1 gene significantly inhibited autophagy after H/R, leading to significantly decreased numbers of autophagosoes and autolysosomes (**Figure 5E**). When looking at the Akt-AMPK pathway, after Beclin 1 knockdown, the expression of p-AKT was significantly downregulated, resulting in a lower p-AKT/AKT ratio (**Figure 5F**, $P < 0.01$). Once re-perfused, the expression of p-AKT was further up-regulated, yielding a higher p-AKT/AKT ratio. The downregulation of the Beclin 1 gene, on the contrast, reduced the expression of p-AKT with a lower p-AKT/AKT ratio (**Figure 5G**, $P < 0.05$). The changes in p-AMPK following Beclin 1 knockdown were similar to these of p-AKT.

Discussion

IRI- mediated PMVEC injury during lung transplantation is considered to be one of the main causes leading to the transplantation failure [2]. We isolated and cultured PMVECs based on the previously reported methods [13, 14], and constructed microvascular endothelial cell injury models through 6 h hypoxia and 2 h reoxygenation. In these models, autophagy was induced and autophagy-related Akt-AMPK signaling pathways were activated. We then used autophagy inhibitor 3-MA to deactivate the Akt-AMPK pathway and inhibit autophagy. We found that apoptosis was decreased, cell viability and migration ability increased. We further examined if Beclin 1 knockdown would deactivate the Akt-AMPK pathway and inhibit autophagy and found results similar to those obtained with 3-MA. These findings indicate that both autophagy inhibitor and knockdown of autophagy-related proteins such as Beclin 1 are able to reduce apoptosis, suggesting that H/R induces autophagy-mediated apoptosis in PMVECs.

Autophagy plays a dual role in determining cell survival and apoptosis. Generally, low level of autophagy helps maintain cellular homeostasis by removing injured proteins and organelles. In adverse environments, such as those resulted from starvation, hypoxia, calcium overload, ROS-induced oxidative stress injury, and endoplasmic reticulum stress, autophagy is strongly induced to degrade proteins to provide essential amino acids to maintain cell survival [15, 16]. However, persistent autophagy is also one of the major mechanisms that regulate apoptosis. Autophagy is thus considered as a programmed cell death [17]. IRI is often accompanied by nutritional deficiency, acidosis, endoplasmic reticulum stress and oxidative stress. These stress responses are shown to be the pathophysiological mechanisms that induce autophagy [18, 19]. In our previous study, it is found that autophagy plays an important role in regulating the function of transplanted rat lung [20], suggesting that autophagy may also be involved in regulating I/R-mediated injury in PMVECs. In addition, many studies have shown that in different experimental models and I/R strategies, the activation of autophagy may play an opposite role in IRI. Inhibition of autophagy reduced the apoptosis after long-term cold ischemia of kidney and improved renal function after transplantation [21]. However, Suzuki et al found that use of 3-MA to inhibit autophagy promoted the apoptosis of renal tubular cells and increased renal IRI [22]. Many studies have confirmed the protective effects of autophagy on cardiac IRI *in vivo* and *in vitro* [23, 24], but others show that high level of autophagy may increase cardiac IRI [25]. In tissues such as heart, brain, liver and kidney IRI is often associated with elevated levels of autophagy. By regulating autophagy level, the acute IRI and apoptosis were regulated successfully [21, 22, 26]. Using rat lung IRI model, we found that 3-MA can inhibit autophagy, reduce autophagy level in lung tissue after I/R, reduce apoptosis and improve lung function, suggesting that I/R increases the autophagy level in lung tissue and excessive autophagy may destroy intracellular homeostasis, induce apoptosis, and aggravate IRI [11]. In this study, we found that autophagy was significantly increased in hypoxia-treated PMVECs and 3-MA remarkably enhanced apoptosis. Meanwhile, knockdown of Beclin-1 inhibited apoptosis, suggesting that during hypoxia, autophagy plays a protective role in cells. H/R treatment also significantly enhanced autophagy in PMVECs, but both 3-MA and Beclin-1 knockdown effectively inhibited apop-

tosis, suggesting that the cell death is mediated by autophagy. These findings demonstrate that autophagy-mediated microvascular injury induced by H/R may be an effective target for improving the efficiency of lung transplantation.

The regulation mechanism of autophagy is very complex and has not been fully elucidated. A number of proteins (Atg) and cellular pathways are involved in the process. The induction of autophagy requires three core complexes, the Atg1 complex, TOR complex 1 (TORC1) and Class III PI3K complex. TORC1 senses the stimulation in cellular nutritional environment: in low nutritional status AMPK is stimulated to be phosphorylated and mTOR is inhibited to activate autophagy [27]. The class III PI3K is required in the formation of autophagosomes [28, 29] and its downstream signal AKT activates Beclin-1 to elevate autophagy level [30]. Beclin-1 interacts with anti-apoptotic protein Bcl-2 to regulate autophagy and apoptosis [31, 32]. The activation of these signal pathways induces partial phosphorylation of Atg13 to bind Atg1, resulting in the activation of Atg1, Atg5 and LC3-II to participate in the formation of autophagosome and lysosome [33, 34]. Soo far, little has been studied regarding autophagy in respiratory system diseases [35], although the high expression of autophagy in lung tissue has been found in chronic respiratory diseases such as smoking, COPD and pulmonary arterial hypertension. The high expression is associated with cell apoptosis, fibrosis and remodeling. It has been predicted that autophagy will become a new frontier in lung disease research [35]. Many studies have shown that nutritional deficiency, lower content of high energy phosphates and elevated ADP/ATP ratio may activate AMPK to regulate autophagy via mTOR. Hypoxia or increased acidity is found to induce the expression of Bnip2 (a member of Bcl-2 family) to activate autophagy. Other factors that activate autophagy include calcium overload, ROS, and opening of mitochondrial permeability transition pore [36]. Therefore, we speculated that IRI should be accompanied by increased autophagy. We observed increased autophagy in lung IRI after acute H/R treatment for 1 h because the expression of LC3-II was found upregulated and autophagic flux was enhanced significantly [11]. Studies have shown that the interaction between anti-apoptotic protein Bcl-2 and autophagy-related protein Beclin-1 and the Akt-AMPK-mTOR signaling pathway may be two important molecular mechanisms regulating protection/injury via autophagy. In myocardial ischemia model, autophagy is deactivated by activation of AMPK and inhibition of mTOR, resulting in protection of cell. However, after reperfusion, AMPK is not activated. Therefore, Beclin-1-mediated non-AMPK pathway may play a role in myocardial injury [37]. We found that the formation of autophagosome was greatly facilitated as a result of higher expression of Beclin-1 and LC3-II after H/R treatment. When the cells were treated with 3-MA, the expression of Beclin-1 and LC3-II was downregulated, leading to inhibition of autophagy. Similar outcomes were observed when Beclin-1 was knockdown. H/I treatment also resulted in down-regulation of Bcl-2 and up-regulation of Bax, yielding an increased Bcl-2/BAX ratio, a direct indicator of apoptosis [38], suggesting that the decreased Bcl-2 causes Beclin1- mediated autophagy that also mediates apoptosis at the same time. In addition, H/R was shown to activate class III PI3K to regulate autophagy [39]. We examined the expression of class III PI3K downstream molecules p-AKT and p-AMPK and found that after H/R treatment, the two molecules were up-regulated significantly. Moreover, it is found that classic class III PI3K inhibitor 3-MA down-regulated the expression of p-AKT and p-AMPK as did Beclin-1 knockdown. These findings indicate that H/R may also regulate autophagy through the activation of the Akt-AMPK pathway.

Taken together, we conclude that H/R induces autophagy and increases apoptosis in PMVECs. The possible mechanisms include the up-regulation of Beclin-1 mediated by H/R-activated class III PI3K downstream signal AKT, autophagy mediated by the activated AMPK-mTOR signaling pathways and apoptosis mediated by imbalanced Beclin-1 and Bax/Bcl-2 expression.

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Disclosure of conflict of interest

None.

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