RESEARCH ARTICLE

Integrin αvβ5 inhibition protects against ischemia-reperfusion-induced lung injury in an autophagy-dependent manner

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Integrin αvβ5 inhibition protects against ischemia-reperfusion-induced lung injury in an autophagy-dependent manner. Am J Physiol Lung Cell Mol Physiol 313: L384–L394, 2017. First published May 18, 2017; doi:10.1152/ajplung.00391.2016.—Integrin αvβ5 mediates pulmonary endothelial barrier function and acute lung injury (LI), but its roles in cell apoptosis and autophagy are unclear. Thus, the aims of this study were to investigate the significance of αvβ5 in ischemia-reperfusion (I/R)-induced apoptosis and LI and to explore the relationship between αvβ5 and autophagy. Human pulmonary microvascular endothelial cells (HPMVECs) were pretreated with an αvβ5-blocking antibody (ALULA) and challenged with oxygen-glucose deprivation/oxygen-glucose restoration, which mimics I/R; then, cellular autophagy and apoptosis were detected, and cell permeability was assessed. In vivo, mice were pre-treated with the autophagy inhibitor chloroquine (CLQ), followed by treatment with ALULA. The mice then underwent operative lung I/R. LI was assessed by performing a pathological examination, calculating the wet/dry lung weight ratio, and detecting the bronchial alveolar lavage fluid (BALF) protein concentration. αvβ5 inhibition promoted HPMVEC autophagy under I/R in vitro, alleviated cell permeability, decreased the apoptosis ratio, and activated caspase-3 expression. These outcomes were significantly diminished when autophagy was inhibited with a small-interfering RNA construct targeting autophagy-related gene 7 (siATG7). Moreover, ALULA pretreatment alleviated I/R-induced LI (I/R-LI), which manifested as a decreased wet/dry lung weight ratio, an altered BALF protein concentration, and lung edema. Preinhibiting autophagy with CLQ, however, eliminated the protective effects of ALULA on I/R-LI. Therefore, inhibiting αvβ5 effectively ameliorated I/R-induced endothelial cell apoptosis and I/R-LI. This process was dependent on improved autophagy and its inhibitory effects on activated caspase-3.

Autophagy is further induced by stresses such as starvation, hypoxic conditions (31), and growth factor depletion (34). Many studies have described its protective effects under pathological conditions and verified the adverse effects of its disruption on cell survival in a diverse array of organisms (11, 15). Our previous work has demonstrated the protective role of autophagy in endothelial progenitor cells under short-term hypoxic conditions (31). Although autophagy is associated with the progression of many pulmonary diseases, such as acute LI and chronic obstructive pulmonary disease (13, 19), its exact role and regulatory mechanisms in I/R-LI remain largely unknown.

Thus, the aims of this study were to investigate the role of αvβ5 in I/R-induced endothelial cell apoptosis and to determine whether this process is dependent on autophagy. Our research and findings may provide new insights into the mechanisms underlying I/R-LI and facilitate new treatments for I/R-LI/Related diseases.
MATERIALS AND METHODS

Chemicals and antibodies. An IgG2b isotype control antibody (CT-Ab; mouse monoclonal antibody to human LDL receptor) was obtained from Mybiosource (MBS310928; San Diego, CA). An integrin αβ3-blocking antibody (ALULA) was kindly provided by Prof. Dean Sheppard (Lung Biology Center, University of California San Francisco). The following reagents were purchased from Sigma-Aldrich: chloroquine (CLQ; C6626), monodansylcadaverine (MDC; 30432), and pentobarbital sodium salt (P3761). Additionally, a BCA Protein Assay Kit was purchased from Beyotime Institute of Biotechnology (P0012). Endothelial cell growth medium (ECM) and Dulbecco’s modified Eagle’s medium (DMEM) (no glucose) were purchased from Sciencell (1001) and Invitrogen (11966-025), respectively. Rhodamine-conjugated phalloidin was purchased from Invitrogen (R415; Molecular Probes). An annexin V-FITC Apoptosis Detection Kit was purchased from BD PharMingen (556547), and a LIVE/DEAD Viability/Cytotoxicity Kit was purchased from Life Technologies (L-3224). A small-interfering RNA construct targeting autophagy-related gene 7 (siATG7), a siRNA transfection reagent system, and an antibody against ATG7 were purchased from Santa Cruz Biotechnology (sc-41447, sc-45064, and sc-33211, respectively). Anti-microtubule-associated protein 1-light chain 3 (LC3) B (3868), anti-questosome 1/p62 protein (p62; 5114), anti-caspase-3 (9662), anti-β-actin (4970), and anti-rabbit IgG (7074) antibodies were purchased from Cell Signaling Technology.

Experimental cells and animals. For in vitro experiments, human pulmonary microvascular endothelial cells (HPMVECs; Sciencell, 3000) were grown to confluence (usually 3–5 days) in ECM and then detached with trypsin-ethylene diamine tetraacetic acid and transferred to new dishes at a split ratio of 1:2 for further propagation. The cells (3–5 passages) were harvested and analyzed. For in vivo experiments, 8- to 10-wk-old wild-type C57BL/6J mice (Animal Center, Fudan University, Shanghai, China) were used. The animals had no access to solid food but were given free access to water 12 h before experiments. The experimental protocol was approved by the Committee of Animal Care of Fudan University. All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals (7).

In vitro grouping and I/R imitation. HPMVECs were pretreated with siATG7. The cells were then randomly divided into a control group with or without CT-Ab or ALULA preconditioning and an in vitro I/R group with or without CT-Ab or ALULA preconditioning. Specifically, 10 μg/ml CT-Ab or ALULA was added into the culture medium at the beginning of the in vitro I/R challenge. An in vitro I/R method was applied to mimic I/R, as previously described (35). The cells were subjected to in vitro I/R by replacing the medium with DMEM lacking glucose and fetal calf serum. Cells were then placed in an anaerobic chamber (5% CO2-95% N2; BioSpherix, C-174) at 37°C for 1 h. The medium was then replaced with an equal volume of medium. The amounts of FITC-dextran in the upper and lower wells of the transwell chambers, samples were taken at 0 and 12 h from the lower compartments of the transwell chambers after in vitro I/R treatment, followed by refilling with an equal volume of medium. The amounts of FITC-dextran in the upper and lower wells were determined using a fluorescence microplate reader (FLX800TBD; BioTek Instruments) at an excitation wavelength of 492 nm and an emission detection wavelength of 520 nm.

Transmission electron microscopy analysis of autophagy ultrastructures. After in vitro I/R treatment, HPMVECs were prefixed with 2.5% glutaraldehyde in 0.1 M PBS at 4°C overnight, postfixed in 1% buffered osmium tetroxide, dehydrated in a graded alcohol series, embedded in Epon 812, sectioned with an ultramicrotome, and stained with uranyl acetate and lead citrate. Cell autophagy precursors, autophagosomes, and autophagolysosomes were observed using transmission electron microscopy (TEM; Philips, CM120). A total of 30 sections were prepared, and autophagy structures were examined in 200 cells from each group.

RNA interference. For siRNA transfection, 2×10⁶ cells were transfected with 60 nM control siRNA (CT-siRNA) or siATG7. After 36 h, the cells were treated with in vitro I/R, and cell death was detected.

Transendothelial permeability assay. FITC-dextran (1 mg/ml, MW 40,000) was added into the medium in the upper wells of the transwell chambers. Samples were taken at 0 and 12 h from the lower compartments of the transwell chambers after in vitro I/R treatment, followed by refilling with an equal volume of medium. The amounts of FITC-dextran in the upper and lower wells were determined using a fluorescence microplate reader (FLX800TBD; BioTek Instruments) at an excitation wavelength of 492 nm and an emission detection threshold of 520 nm. Final values were determined by subtracting the fluorescence values at 0 h. The results are presented as a percentage of the fluorescence in the control group.

Cell viability assay. A LIVE/DEAD Viability/Cytotoxicity Kit was used to measure cell viability. Briefly, cells were cultured on sterile glass coverslips as confluent monolayers. Then, 20 μl of 2 mM ethidium homodimer (EthD)-1 was added to 10 ml of PBS and then combined with 5 μl of a 4-mM calcein AM solution. The working solution, which contained 2 μM calcein AM and 4 μM EthD-1, was directly added to the cells. After 15 min, the cells were examined using a confocal laser-scanning microscope.

Apoptosis detection by flow cytometry. According to the instructions for the Annexin V-FITC Apoptosis Detection Kit, cells were washed twice with cold PBS and then resuspended in 1× binding buffer at a concentration of 1×10⁶ cells/ml. One hundred microliters of solution was transferred to 5-ml culture tubes, followed by the addition of 5 μl of annexin V-FITC and 5 μl of propidium iodide. The resulting solution was incubated for 15 min at room temperature in the dark, followed by the addition of 400 μl of 1× binding buffer. Apoptosis rates were immediately analyzed by performing flow cytometry (FCM; BD Biosciences, FACS Aria III).
Establishment of a mouse model of lung I/R and study groupings. Animals were randomly assigned to a group that underwent sham operations with or without CLQ or ALULA pretreatment or a group that underwent lung I/R operations with or without CLQ or ALULA pretreatment. Pretreatment was applied using the following doses and approaches: CLQ was intraperitoneally injected at 10 mg/kg into animals 4 h before I/R, and ALULA was intravenously injected at 4 mg/kg immediately before the experiment in the appropriate experi-

Fig. 1. The effects of αvβ5-blocking antibody (ALULA) on the autophagy levels in in vitro ischemia-reperfusion (I/R)-challenged human pulmonary microvascular endothelial cells (HPMVECs). A: representative Western blot images of microtubule-associated protein 1-light chain 3 (LC3) and sequestosome 1/p62 from the ALULA or isotype control antibody for ALULA (CT-Ab) preconditioned groups and statistical analysis. B: changes in autophagic structures and stress fiber formation were detected by staining for LC3 and F-actin; statistical analyses were carried out to quantitate the no. of LC3-positive puncta. Green spots represent LC3-positive puncta. Results are given as means ± SE (n = 5), and the molecular mass is given (in kDa). p62, sequestosome 1/p62.
mental groups \((n = 6/\text{group/detection index})\) \((8, 25)\). Operations were performed as previously described with modifications \((26)\). The mice were anesthetized via the intraperitoneal administration of 60 mg/kg pentobarbital sodium salt and the intramuscular injection of atropine at 0.06 mg/kg. After cannulation, the mice were placed on a Harvard ventilator \((\text{Harvard Apparatus Inspira ASV, 55-7059})\), with room air at a tidal volume of 0.28 ml and a respiratory rate of 85/minute. The animals were placed on their right sides to allow a left anterolateral thoracotomy in the fifth intercostal space to be performed. The left hilum was cross-clamped for 30 min, after which the cross-clamp was released for 120 min. Lung specimens were obtained for further investigation.

Lung tissue morphology and edema detection. Fresh left lung tissues were harvested and immediately fixed in 10% formalin and then embedded in paraffin. Four-micrometer-thick paraffin sections were stained with hematoxylin and eosin. LI was assessed in terms of four categories: interstitial inflammation, neutrophil infiltration, congestion, and edema. The results are expressed as scores on a 0- to 4-point scale: no injury = score of 0; injury in 25% of the field = score of 1; injury in 50% of the field = score of 2; injury in 75% of the field = score of 3; and injury throughout the field = score of 4 \((21)\). Ten microscopic fields from each slide were analyzed, and the scores of the fields were averaged to evaluate LI severity. All...

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**Fig. 2.** The effects of ALULA on autophagic ultrastructures of HPMVECs. **A:** representative transmission electron microscopy (TEM) images of autophagic ultrastructures. N represents the cell nucleus. **B:** quantitative analysis of the ratios of cross-sectional areas of autophagic structures to that of the cytoplasm. Autophagosomes and autolysosomes are indicated by arrowheads and arrows, respectively. The results are given as means ± SE \((n = 5)\).
microscopic sections were interpreted in a blinded fashion by a pulmonary pathologist.

In addition, lung edema was assessed by examining the lung wet-to-dry weight (W/D) ratio. After the lung wet weight was measured, the same lung was dried at 56°C for 72 h. The W/D ratio was then calculated.

Detection of the total protein concentration in bronchoalveolar lavage fluid. After reperfusion, the left lungs were lavaged with 0.5 ml of saline via tracheotomy. This procedure was performed three times, and the fluid was pooled together. An average of 1.2 ml of total bronchoalveolar lavage fluid (BALF) was collected from each mouse and centrifuged at 4°C (1,000 rpm for 15 min). The supernatants were

Fig. 3. Inhibition of autophagy-related gene 7 (ATG7) expression weakened the effects of ALULA on autophagy in in vitro I/R-challenged HPMVECs. A: representative Western blot image and statistical analysis of ATG7 expression after treatment with small-interfering RNA targeting autophagy-related gene 7 (siATG7). B: fluorescent images and corresponding statistical analysis for monodansylcadaverin (MDC)-labeled autophagosomes in cells with or without siATG7 preconditioning. Green puncta mark MDC-labeled autophagosomes. Results are given as means ± SE (n = 5).
collected and stored at −80°C, and the BALF protein concentration was detected using a BCA Protein Assay Kit.

Statistical analyses. The data were presented as means ± SE and analyzed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). One-way analysis of variance was performed to compare multiple groups, followed by the Bonferroni post hoc test for intergroup comparisons. Histological semiquantitative comparative analyses were performed using the nonparametric Mann-Whitney test. A P value of <0.05 was considered significant.

RESULTS

Integrin αβ5 inhibition improved autophagy levels in HPMVECs under in vitro I/R. To study the role of αβ5 in autophagy regulation under simulated I/R conditions, we inhibited αβ5 function with ALULA, a specific inhibitor that exclusively recognizes and inhibits αβ5 in mouse, human, and bovine tissues (25). In addition, LC3 and p62 were investigated to detect autophagy levels. LC3 is an autophagosomal ortholog of yeast autophagy-related gene 8. During autophagy, a cytosolic form of LC3-I is lipidated to form LC3-II, which is required for the formation of autophagosome membranes; thus, the conversion of LC3-I to LC3-II is an indicator of autophagy (28). Additionally, LC3-I is used as an indicator of autophagy levels, and LC3-II is used to evaluate autophagy flux (18). As shown, in vitro I/R significantly increased the conversion of LC3-I to LC3-II and decreased p62 expression. Inhibiting αβ5 with ALULA further enhanced autophagy in vitro I/R-treated cells, which was reflected in the further conversion of LC3-I to LC3-II and decreased p62 expression. CT-Ab, however, did not enhance in vitro I/R-induced autophagy. Inhibiting αβ5 with ALULA further enhanced autophagy in vitro I/R-treated cells, which was reflected in the further conversion of LC3-I to LC3-II and decreased p62 expression. CT-Ab, however, did not enhance in vitro I/R-induced autophagy (Fig. 1A). We observed round or oval LC3-labeled autophagic structures. The number of LC3-positive puncta in vitro I/R-challenged cells was greater than that in the control group but lower than that in the ALULA-preconditioned group. Additionally, we observed significant in vitro I/R-induced stress fiber formation that was markedly attenuated by ALULA (Fig. 1B). These findings were consistent with the confirmed effects of ALULA on αβ5 reported in previous studies (24, 25).

The effects of ALULA on autophagic ultrastructures in in vitro I/R-treated cells. Autophagic ultrastructures were examined by TEM. Normal-looking organelles were observed in control cells, whereas representative autophagic structures in in vitro I/R-treated cells are shown in Fig. 2A. Autophagosomes are double- or multimembrane structures that engulf cytoplasmic fractions. In autophagolysosomes, the lysosome contains an autophagosome with a single membrane. In the ALULA pretreatment group, more numerous and larger autophagolysosomes were detected in in vitro I/R-challenged cells. Statistically, the ratios of cross-sectional areas of autophagic structures to cytoplasm in the in vitro I/R groups were significantly higher than those in the control groups but lower than those in the in vitro I/R-challenged and ALULA-preconditioned group (Fig. 2B).

SiATG7 treatment weakened the effects of ALULA on autophagy. To determine whether the effects of ALULA on in vitro I/R-challenged cells were dependent on autophagy, we applied a specific siRNA to reduce autophagic activity by inhibiting the expression of ATG7, an essential autophagy molecule involved in the conversion of LC3-I to LC3-II and in autophagosome formation (10). As shown, siATG7 treatment not only effectively inhibited the expression of ATG7 (Fig. 3A) but also markedly prevented the formation of autophagosomes induced by in vitro I/R or ALULA (Fig. 3B).

Autophagy inhibition counteracted the effects of ALULA on HPMVEC permeability. To determine whether autophagy is involved in the process by which αβ5 regulates HPMVEC barrier integrity, we measured cell permeability under different culture conditions. As shown, cell permeability in the in vitro I/R-challenged group was significantly increased compared with that in the control group. Inhibiting αβ5 or ATG7 effectively decreased or aggravated, respectively, the high permeability induced by in vitro I/R. In addition, cell permeability was much higher in ALULA-preconditioned and siATG7-treated cells than in cells that were not treated with siATG7 (Fig. 4).

The antiapoptotic effects of ALULA on in vitro I/R-challenged HPMVECs were autophagy dependent. A LIVE/DEAD Viability/Cytotoxicity kit was used to investigate cell death. In normal endothelial cells, in vitro I/R induced serious cell death, which was characterized by ruptured cell membranes and shrinking nuclei. Inhibiting αβ5 with ALULA markedly alleviated in vitro I/R-induced cell death. However, in cells treated with siATG7, in vitro I/R led to more serious cell death than that observed in wild-type cells. Inhibiting αβ5 did not further alleviate in vitro I/R-induced cell death in these cells (Fig. 5A). Additionally, FCM was performed to test the effects of ALULA on cell apoptosis. In wild-type cells, in vitro I/R significantly triggered cell apoptosis, but this response was markedly restrained when αβ5 was inhibited with ALULA. However, when autophagy was inhibited with siATG7, in vitro I/R induced greater levels of cell apoptosis than those observed in wild-type cells. Furthermore, ALULA preconditioning did not alleviate in vitro I/R-induced apoptosis in these cells (Fig. 5B). We next applied control antibody to detect whether the antiapoptotic effects of ALULA were derived from its role in blocking αβ5 or other off-target effects. As shown, the control antibody did not exert additional effects on in vitro I/R-induced apoptosis (Fig. 5C).

Fig. 4. Autophagy inhibition counteracted the effects of ALULA on in vitro I/R-induced endothelial cell permeability. Statistical analysis of cell permeability with or without siATG7 preconditioning. The results are given as means ± SE (n = 5).
Fig. 5. Autophagy inhibition influenced the effects of ALULA on the survival and apoptosis of in vitro I/R-challenged HPMVECs. A: representative fluorescent images and statistical analysis of cell death in in vitro I/R-challenged cells with or without sequential siATG7 and ALULA preconditioning. Live cells with intact cellular membranes fluoresced bright green (arrow). Dead cells with compromised membranes fluoresced orange (arrow head). B: typical flow cytometry (FCM) quadrantal diagrams and corresponding statistical data for apoptotic cells in the ALULA-preconditioned group. C: typical FCM quadrantal diagrams and corresponding statistical data for apoptotic cells in the CT-Ab-preconditioned group. Results are given as means ± SE (n = 5).
The inhibitory effects of ALULA on caspase-3 activity were autophagy dependent. Activated cleaved caspase-3 expression was examined to further clarify the role of αvβ5 in apoptosis. Caspases are crucial mediators of apoptosis. Among them, activated caspase-3 is vital and correlates positively with apoptosis (33). As shown, in vitro I/R significantly increased cleaved caspase-3 (activated caspase-3) expression levels, but this effect was markedly weakened or enhanced by αvβ5 or autophagy inhibition, respectively. Furthermore, cleaved caspase-3 expression in cells pretreated by siATG7 was measured to clarify autophagy involvement in regulating the effects of ALULA. As shown, autophagy inhibition eliminated the effects of ALULA on cleaved caspase-3 expression under in vitro I/R conditions (Fig. 6).

ALULA protected against I/R-induced alveolar capillary membrane barrier damage by regulating autophagy. To determine whether the effects of ALULA on the integrity of the alveolar capillary membrane barrier were autophagy dependent, autophagy was inhibited in mice via the application of CLQ, a lysosomotropic drug that raises the intralysosomal pH and impairs autophagic protein degradation, thus effectively inhibiting the last step in the autophagic degradation process (2). In addition, we determined the severity of overall lung edema and damage to the alveolar capillary membrane barrier by detecting the lung W/D weight ratio. As shown, CLQ or ALULA pretreatment did not change the lung W/D weight ratio in the sham operation group. I/R significantly increased the lung W/D weight ratio compared with that in the sham operation group, whereas this effect was mitigated by treatment with ALULA. However, CLQ preconditioning eliminated the effects of ALULA on the W/D weight ratio under lung I/R conditions (Fig. 7A).

BALF protein concentration is another indicator of lung alveolar-capillary membrane permeability. There were no significant differences in total BALF protein concentration between the sham groups with or without ALULA and CLQ pretreatment. Lung I/R significantly increased the total BALF protein concentration compared with that in sham animals, whereas the BALF protein concentration was notably reduced by ALULA pretreatment. However, autophagy inhibition using CLQ nearly completely suppressed the effects of ALULA on the total BALF protein concentration (Fig. 7B).

The effects of ALULA on I/R-LI were autophagy dependent. Lung tissue edema, leukocyte infiltration, and hemorrhage were comprehensively assessed, and ALULA and CLQ did not exert extra influence on mice undergoing sham operations. However, inhibiting αvβ5 with ALULA markedly alleviated I/R-LI, and preinhibiting autophagy with CLQ nearly counteracted the effects of ALULA on I/R-LI (Fig. 8A). Statistically, I/R increased LI scores, a phenomenon that was alleviated by inhibiting αvβ5. However, CLQ preinjection eliminated the effects of ALULA on LI scores in I/R-challenged mice (Fig. 8B)

DISCUSSION

During lung I/R, pulmonary cellular apoptosis is induced by a variety of factors, such as reactive oxygen species, and plays a key role in hastening pulmonary failure (16). To initiate apoptosis, extracellular stimuli must be transmitted into cells. This process directly or indirectly involves interactions with integrins (12). Certain harsh LI conditions, such as ischemia, pneumonia, and sepsis, affect integrin-extracellular matrix interactions, which affect the ligation or configuration of those integrins, thus altering their functions. Integrin molecules likely have roles in apoptosis, but whether and how integrin αvβ5 is involved in HPMVEC apoptosis under I/R remains unclear. The aims of this study were thus to investigate whether αvβ5 was involved in lung I/R-induced endothelial cell apoptosis and to explore the role of autophagy in this process.

Although some researchers have demonstrated antiapoptosis responses for certain integrin subtypes (17), our study is the first to verify the involvement of αvβ5 in lung I/R-triggered apoptosis and caspase-3 activation. Some integrins promote cellular survival and induce apoptosis when inappropriately ligated or left unligated (23). According to Varner et al. (29), the cytoplasmic domain of the integrin β-subunit is proapoptotic and sufficient to induce apoptosis, even in attached cells. Thus, different integrins may possess different capacities to induce apoptosis due to the presence of regulatory regions elsewhere in the integrin heterodimer. In addition, these inconsistent results may stem from different configurations for the same integrin subtype under various pathological conditions. Therefore, further studies are necessary to characterize the effects of different configurations of αvβ5 on apoptosis.
We identified a regulatory role for $\alpha_\beta 5$ in lung I/R-induced apoptosis in our study, and therefore, we deduced that $\beta_5$ may influence autophagy, a crucial coordinator during stress-induced apoptosis. After autophagy was inhibited using si-ATG7, in vitro I/R-induced cell apoptosis was significantly increased but was not efficiently blocked by ALULA. Thus, autophagy affects the regulation of $\alpha_\beta 5$ during apoptosis. To further elucidate the relationship between $\alpha_\beta 5$ and autophagy, we detected autophagy levels in in vitro I/R-challenged endothelial cells preconditioned with or without ALULA and observed improved in vitro I/R-induced autophagy with $\alpha_\beta 5$ inhibition. This finding was likely attributable to the ability of ALULA to inhibit $\alpha_\beta 5$ but not its off-target effects. Previous studies have also demonstrated autophagy induction following integrin blockade. According to He et al. (5), knockdown of integrin $\beta_4$ induced autophagy in A549 cells. Vom Dahl et al. (30) reported significant elimination of the hypoosmolarity-induced decrease in liver cell autophagic vacuoles following hexapeptide-mediated integrin inhibition. In addition, the downregulation of $\beta_3$-integrin results in enhanced LC3-II and Beclin-1 expression during heart failure (27). However, according to other studies, integrin blocking reportedly inhibits the autophagy process. In a study by Edick et al. (3), blocking integrins $\alpha_3$, $\alpha_6$, or $\beta_4$ with antibodies inhibited autophagy in human primary basal prostate epithelial cells. Zheng et al. (36) also reported significantly diminished osteopontin-induced autophagosome formation in smooth muscle cells following integrin/CD44 blockade. These data implicate dual roles for integrins in both the suppression as well as the stimulation of autophagy. These seemingly contradictory findings may be attributable to different model systems and different culture conditions. However, these studies highlight the complex relationships between different integrins and autophagy. Therefore, further studies are necessary to clarify the correlation between a specific type of integrin and autophagy.

In this study, we have demonstrated exacerbated in vitro I/R-induced apoptosis and elimination of the antiapoptotic functions of ALULA with autophagy inhibition. These findings reflect the significant inhibitory effects of autophagy on apoptosis. Similar results have been reported in previous studies. Boya et al. (1) observed HeLa cell apoptosis following autophagy blockade at an early stage due to cellular failure to adapt to starvation conditions. Similarly, Beclin-1 depletion during Caenorhabditis elegans development increases the number of apoptotic cell corpses (27). Many researchers have focused on the caspase cascade, a critical regulator that initiates and sustains apoptosis, when examining the regulatory mechanisms of autophagy in apoptosis (32). Hou et al. (6) observed increased caspase-8 activity and the elimination of large caspase-8 subunit sequestration in autophagosomes to lysosomal degradation following autophagy inhibition. Recently, Han et al. (4) reported the ability of ATG7 to repress the apoptotic capability of caspase-9. In our current study, autophagy inhibition increased caspase-3 activation in vitro I/R-challenged cells. Given the critical role of activated caspase-3 as an “effector” caspase associated with initiation of the “apoptosis cascade,” we suspect it is one of...
the targets for autophagy in regulating lung I/R-induced apoptosis. Further studies will be carried out to explore the exact regulatory mechanisms of autophagy in caspase-3 activation.

The present study demonstrated the mitigation of I/R-induced endothelial cell apoptosis and I/R-LI following \( \alpha \nu \beta 5 \) inhibition with the specific antibody ALULA; specifically, \( \alpha \nu \beta 5 \) inhibition improved autophagy, which retarded caspase-3 activation (Fig. 9). The findings reported here have potential clinical relevance; \( \alpha \nu \beta 5 \) and autophagy appear to be attractive therapeutic targets for lung I/R-related diseases such as lung transplantation and cardiopulmonary bypass.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS

D.Z. and C.L. performed experiments; D.Z., C.L., Y.L., and C.B. analyzed data; D.Z., C.L., and J.Z. interpreted results of experiments; D.Z., C.L., and J.Z. prepared figures; D.Z., C.L., and C.B. drafted manuscript; D.Z., C.L., J.L., and C.B. edited and revised manuscript; D.Z., C.L., Y.S., J.Z., Y.L., J.L., and C.B. approved final version of manuscript; D.Z. conceived and designed research.
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