High shear stress-induced pulmonary hypertension alleviated by endothelial progenitor cells independent of autophagy

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Background: **Pulmonary hypertension (PH) is a progressive disease characterized by lung endothelial cell dysfunction and vascular remodeling. Endothelial progenitor cells (EPCs) have been proved to be a potential therapeutic strategy to treat PH. Autophagy has been found to be protective to hypoxia-induced PH. In this study, we applied high shear stress (HSS)-induced PH, and examined whether EPCs confer resistance against HSS-induced PH through autophagy.**

Methods: **Pulmonary microvascular endothelial cells (PMVECs) were cultured under HSS with pro-inflammatory factors in an artificial capillary system to mimic the PH condition. Levels of p62, a selective autophagy substrate, were quantified by western blotting. Cell viability was determined by trypan blue exclusion test.**

Results: **The p62 level in PMVECs was increased at 4 hours after HSS, peaked at 12 hours and declined at 24 hours. The cell viability gradually decreased. Compared with PMVECs cultured by empty medium, in cells cultured by EPC-conditioned medium (EPC-CM), the cell viability was significantly higher; however, p62 levels were also significantly higher, suggesting inhibition of autophagy by EPC-CM. Adding choloquine to suppress autophagy decreased the cell viability of PMVECs under PH.**

Conclusions: **EPC-CM could suppress the autophagic activity of PMVECs in HSS-induced PH. However, suppression of autophagy leads to cell death. EPCs could fight against PH through cellular or molecular pathways independent of autophagy. But it is not proved if induction of autophagy could be a potential strategy to treat HSS-induced PH as hypoxia-induced PH.**

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Introduction

Pulmonary hypertension (PH) is a condition with the mean pulmonary arterial pressure of more than 25 mmHg at rest or 30 mmHg during exercise.[1] PH is classified into 5 categories, including pulmonary arterial hypertension, PH secondary to left heart disease, PH secondary to lung diseases and/or hypoxia, chronic thromboembolic pulmonary hypertension and PH with unclear multifactorial mechanisms.[2] Hypoxic PH is the most studied model of PH, which can also be mimicked by introducing high shear stress (HSS) plus pro-inflammatory factors in the artificial capillary system.[3] Although the molecular and cellular mechanisms underlying the vascular changes associated with PH are still unclear,[4] impairment of vascular and endothelial homeostasis is thought to play an important role during the initiation and development of PH.[5] Once developed, PH is irreversible and progressive with a poor prognosis.

Endothelial progenitor cells (EPCs) have the capacity to circulate, proliferate, and differentiate into mature endothelial cells. In recent years, some evidences including a pilot trial in patients[6] have suggested that autologous transplantation of EPCs

has beneficial effects on exercise capacity and pulmonary hemodynamics, providing novel ideas to treat PH. In hypoxic PH, the beneficial effect of EPCs transplantation has been mostly attributed to vasculogenesis, vascular repair and regeneration due to the stemness characteristic of EPCs.[7]

Autophagy is a fundamental cellular physiological process that functions in the turnover of subcellular organelles and macromolecules.[8] As a cell survival pathway,[9] autophagy is connected to a number of human diseases, including cancer,[10] neurodegenerative diseases,[11] heart disease,[12] inflammatory bowel disease,[13] and chronic lung disease.[14] In hypoxia-induced PH, it was reported that the cellular autophagic activity was increased in the lung and lung vasculature of patients, and increased autophagy was shown to play a protective role by *in vitro* models.[9] Therefore, we hypothesized that EPCs could confer resistance against HSS-induced PH through modulating autophagy. To test this hypothesis, an *in vitro* study was performed to determine the autophagy activity in pulmonary microvascular endothelial cells (PMVECs) cultured under HSS stimulation in either empty medium (EM) or EPC-conditioned medium (EPC-CM) from EPCs cultures.

Methods

EPCs isolation and culture

Male Sprague-Dawley rats (250-350 g of weight, 8 weeks) were purchased from Zhejiang University Animal Research Center. After rats were euthanized using CO_2, bone marrow-derived EPCs were isolated, purified and cultured *in vitro* as described previously.[15] EPCs were confirmed by Dil-acetylated low-density lipoprotein (Dil-Ac-LDL) (Molecular Probes, Eugene, OR, USA) uptake and fluorescein isothiocyanate conjugated ulex europaeus agglutinin-1 (FITC-UEA-1) (Sigma, St Louis MO, USA) binding. All animal studies were performed according to the principles of laboratory animal care and were approved by the Animal Ethics Committee of Zhejiang University.

Preparation of CM and EM

EPC-CM was collected as previously described.[16] On the 5th day of culture, EPCs were obtained and cultured on six-well culture dishes at a density of 5×10^6 cells/well. Twenty-four hours later, the medium was replaced with fresh medium M199 with no supplement (1.5 mL/well). After another 24 hours, culture medium was collected and concentrated (10×) by centrifugation for 20 minutes at 5000×g and 4°C using Ultrafree-4 centrifugal filter tubes with Biomax-5 membranes (Millipore, Billerica, MA, USA). Fresh medium M199 with no supplement was concentrated directly in the same way as the EM.

Pulsatile flow system to produce high shear stress

The FiberCell artificial capillary cell culture system (Frederick, MD, USA) was used to mimic the PH condition *in vitro*, which can provide HSS. The use of this device was based on the method established by Hahn et al.[3] In brief, rat PMVECs (5×10^6/well) (Clonetics, Baltimore, MD, USA) were cultured at a flow rate that provided a shear stress averaging 1.9 dyn/cm^2 in each capillary. Seventy-two hours later, the original medium was discarded and PMVECs were re-incubated with fresh medium M199 with 20% fetal bovine serum. And then HSS of 10.9 dyn/cm^2 was applied. PMVECs were collected at 0, 4, 8, 12, and 24 hours after HSS for further assays including western blotting and cell viability test. On the other hand, HSS was applied when the original medium was replaced with EPC-CM or EM after 72 hours. After another 24 hours, PMVECs were collected to be used.

Western blotting

A total of 20 μg protein from each sample was separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat milk in phosphate buffer solution containing 0.05% Tween before incubation with primary antibodies, followed by horse reddish peroxidase-conjugated secondary antibodies (Santa Cruz, USA). Primary antibodies included anti-β-actin (Sigma, USA) and anti-p62 (Abcam, Cambridge, UK). Films were developed by chemiluminescence kit (Perkin Elmer, Boston, MA, USA).

Cell viability assay

PMVECs were trypsinized after HSS by incubation with trypsin-ethylene diamine tetraacetic acid at 37°C for 5 minutes. The viability of the cells was determined by trypan blue exclusion test using a hemocytometer.

Statistical analysis

All data are presented as mean±standard deviation. The Kolmogorov-Smirnov test was used to study the distribution of the variables. The difference between groups was evaluated statistically by one-way ANOVA followed by Tukey's procedure for post-hoc comparison. $P<0.05$ was considered statistically significant and $P<0.01$ was considered highly significant. All statistical analyzes were performed by using SPSS 16.0 for Windows.

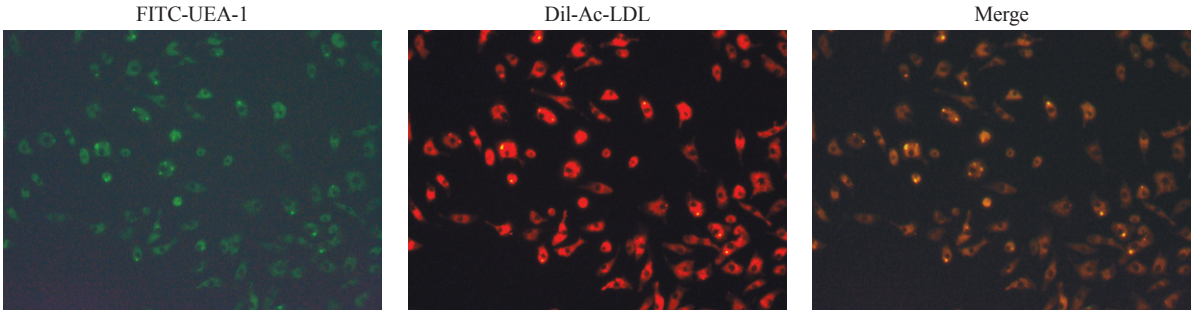

Fig. 1. Isolation of EPCs. EPCs were isolated from the bone marrow of Sprague-Dawley rats and confirmed by Dil-Ac-LDL uptake and FITC-UEA-1 blinding. Typical EPCs with double labeling are shown. EPCs: endothelial progenitor cells; Dil-Ac-LDL: Dil-acetylated low-density lipoprotein; FITC-UEA-1: fluorescein isothiocyanate conjugated ulex europaeus agglutinin-1.

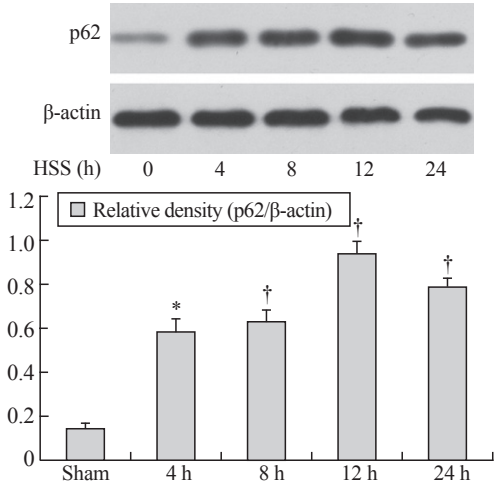

Fig. 2. Suppression of autophagy in PMVECs during HSS-induced PH. A time-course change of p62 in rat PMVECs is shown. PMVECs were collected at 0, 4, 8, 12, and 24 hours after HSS. The p62 protein was quantified by western blotting analysis. β-actin was used as a loading control. Data are shown as mean±SD. PMVECs: pulmonary microvascular endothelial cells; HSS: high shear stress; PH: pulmonary hypertension; SD: standard deviation. *: P<0.05; †: P<0.01, compared with the sham group.

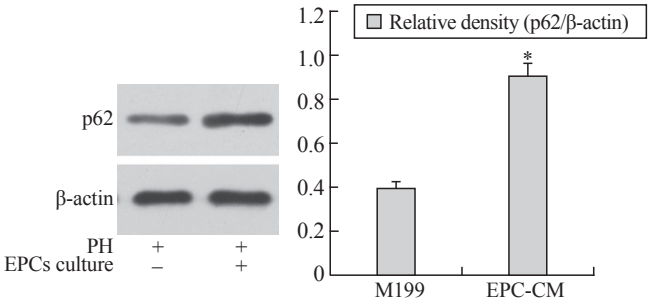

Fig. 3. Suppression of autophagy in EPC-CM-cultured PMVECs during HSS-induced PH. PMVECs were cultured under HSS and with inflammatory factors to mimic PH, empty medium (M199) or EPC-CM was used. After 24 hours of culture, cells were collected, and the levels of p62 and β-actin were determined by western blotting. Statistical analysis was performed by the densitometry of 3 repeated blottings. Data are shown as mean±SD. EPCs: endothelial progenitor cells; EPC-CM: endothelial progenitor cell-conditioned medium; PMVECs: pulmonary microvascular endothelial cells; HSS: high shear stress; PH: pulmonary hypertension; SD: standard deviation. *: P<0.01.

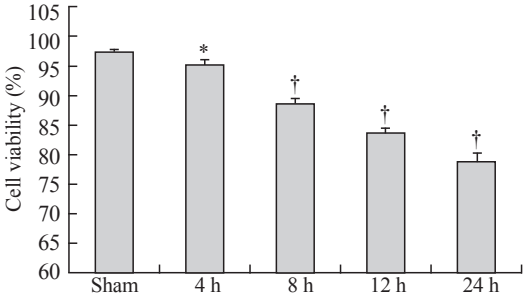

Fig. 4. The time-course cell viability in PMVECs during HSS-induced PH. PMVECs were cultured under HSS, and with inflammatory factors to mimic PH and M199 medium was used. Cell viability after 4, 8, 12 and 24 hours of HSS stimulation was determined by trypan blue exclusion. Data from 3 repeated cultures are presented as mean±SD. PMVECs: pulmonary microvascular endothelial cells; HSS: high shear stress; PH: pulmonary hypertension; SD: standard deviation. *: P<0.05; †: P<0.01, compared with the sham group.

Results

Isolation of EPCs from rat bone marrow

The cells showing Dil-Ac-LDL uptake and FITC-UEA-1 blinding were considered as EPCs based on the previous publication (Fig. 1).[15] Following the procedure, we have successfully isolated a good number of relatively pure EPCs from the bone marrows of rats.

Time-dependent suppression of cellular autophagic activity under HSS

To determine the time-course change of autophagy, we exposed PMVECs under HSS to mimic PH. PMVECs were collected at 0, 4, 8, 12, and 24 hours after HSS. The levels of p62, the autophagy-selective degradation substrate, were quantified by western blotting (Fig. 2). The results revealed that the basal level of p62 was lowered, and after HSS, the p62 protein was increased significantly at 4 hours (P<0.05), peaked at 12 hours (P<0.01) and declined at 24 hours (P<0.01). Increased

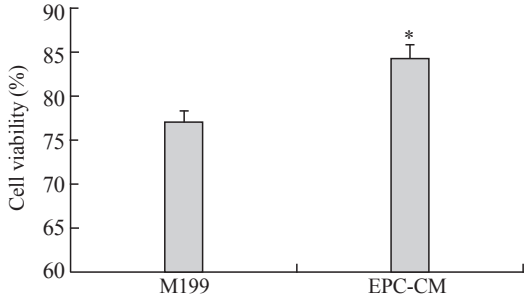

Fig. 5. EPC-CM resulted in increased cell viability. PMVECs were stimulated by HSS and inflammatory factors to mimic PH and cultured in M199 and EPC-CM, respectively. Cell viability was determined after 24 hours of culture and data from 3 repeated cultures are shown as mean±SD. EPC-CM: endothelial progenitor cell-conditioned medium; PMVECs: pulmonary microvascular endothelial cells; HSS: high shear stress; PH: pulmonary hypertension; SD: standard deviation. *: $P<0.05$.

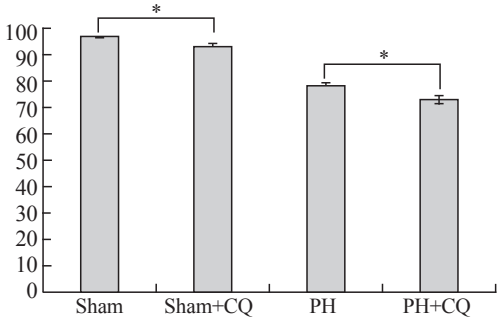

Fig. 6. Inhibition of autophagy by CQ decreased cell viability of PMVECs. PMVECs were cultured with or without (sham) PH stimulation by HSS and inflammatory factors and supplemented with or without CQ at 50 μmol/L. Cell viability was determined after 24 hours of culture in M199 medium. Data from 3 repeated cultures are presented as mean±SD. CQ: chloroquine; PMVECs: pulmonary microvascular endothelial cells; PH: pulmonary hypertension; HSS: high shear stress; SD: standard deviation. *: $P<0.05$.

p62 level is generally considered a marker of suppressed autophagy.[17]

Autophagy suppressed by EPC-CM in PMVECs under HSS

As shown in Fig. 3, in PMVECs under HSS, compared with EM, the p62 protein level was brought up significantly by EPC-CM after 24 hours of culture ($P<0.01$).

Protective role of EPC-CM and autophagy inhibition detrimental to PMVECs under HSS

The time-course cell viability of PMVECs is shown in Fig. 4. As the duration of HSS lasted, cell viability declined gradually from 100% to 95.8%±0.72% at 4 hours, 89.1%±0.83% at 8 hours, 84.2%±0.72% at 12 hours, and 79.3%±1.42% at 24 hours.

Under PH, the cell viability of PMVECs cultured by EPC-CM was significantly higher than that of PMVECs cultured with M199 medium, suggesting a protective role of EPC-CM (Fig. 5). When autophagy was suppressed by chloroquine (CQ), a lysosome inhibitor, either with or without HSS stimulation, the cell viability of PMVECs decreased significantly (Fig. 6).

Discussion

Autophagy is a physiological self-degradation process to maintain cellular homeostasis. Cell stress, including hypoxia, nutrient deprivation or reduction in growth factor stimulation, can stimulate autophagy responses.[18] Autophagy deregulation is involved in the pathogenesis of many diseases including PH.[19] Lee and his colleagues[9] reported an upregulation of autophagy in PH patients compared with healthy controls. Mice with hampered autophagy showed serious PH symptoms, which suggest the potentially protective role of autophagy. However, discrepancy still exists in this field. In a fetal lamb model with persistent PH, cellular autophagic activity in PMVECs was increased; however, down-regulation by 3-methyladenine resulted in decreased autophagy and promoted *in vitro* angiogenesis. Hence autophagy was proved to be detrimental to PMVECs during PH.[20] Our data showed that the cell viability of PMVECs cultured under HSS-induced PH was gradually declined as PH persisted, but autophagy was down-regulated in a time-dependent fashion, as manifested by accumulation of p62, an autophagy substrate and a common marker for autphagy activity.[17] The level of p62 increased gradually during the first 12 hours; however, the level decreased at 24 hours compared with 12 hours, suggesting that autophagy could be activated again as HSS-induced PH persists. Thus, whether autophagy is eventually up-regulated during long-term HSS remains to be investigated.

The above observations indicated inhibition of autophagy as a general response to HSS-induced PH, which was opposite to hypoxia-induced PH models, in which autophagy was found to be activated.[9] However, as discussed above, it remains obscure whether autophagy up-regulation is protective to PH or not. According to the current observation, we hypothesize that autophagy may be a cellular defense against HSS-induced PH and activation of autophagy may increase cell viability. The role of autophagy in PH remains inconclusive, and the mechanisms by which autophagy affects the initiation and progression of PH should be further investigated. Currently, we are investigating whether promotion of autophagy by chemical reagents, such as rapamycin would protect PMVECs from HSS-

induced cell death.

Endothelial injury is essential in the development of PH,[21] which is characterized by cellular and structural changes of pulmonary artery walls, resulting in endothelial dysfunction and vascular remodeling.[22] EPCs are thought to be important in maintaining vascular homeostasis by homing to sites of vascular injury and regenerating blood vessels.[23] In animal models and pilot studies in patients, EPC transplantation was proved to alleviate PH symptoms and pathological changes, providing a novel treatment strategy. Wang et al[6] reported that transplantation of autologous EPC can significantly improve exercise capacity and pulmonary hemodynamics in PH patients. Schiavon et al[24] found an increased number of EPCs at end-stage pulmonary disease, suggesting an involvement in pulmonary vascular remodeling. The mechanism of EPC treatment is not totally unveiled. Elucidating the relevant cellular events and molecular pathways such as autophagy may help to identify new targets and to develop adjuvant therapeutic strategies that improve the efficacy of EPCs transplantation.

In this study, we observed that EPC-CM protected PMVECs in culture under HSS stimulation, suggesting that EPCs may produce some cytokines to protect PMVECs in HSS-induced PH. Furthermore, we examined whether autopahgy regulation is involved in the protective mechanism of EPC-CM culture. Interestingly, application of EPC-CM suppressed autophagy in PMVECs, characterized by accumulation of p62. It is considered that induction of autophagy is cytoprotective in PH. Furthermore, we used lysosome inhibitor CQ to suppress autophagy in cultured PMVECs, and cell viability was decreased by CQ treatment, suggesting inhibition of autophagy is harmful to PMVECs. Our data indicated suppression of autophagy by certain ingredients secreted by EPCs, which may confer resistance against PH through certain cellular or molecular pathways other than autophagy.

Autophagy could be a promising target for PH treatment as for other conditions currently under clinical trials, including cancer, viral infection, and neurodegenerative diseases.[25,26] Current data support that autophagy actively participates in EPC-mediated vascular remodeling during PH.[27,28] However, the direct effects of autophagy on vascular epithelial cells and smooth muscle cells are more complicated. From the literatures, autophagy activation is toxic to PMVECs,[20] but promotes the survival of arterial smooth muscle cells.[29] Although we found that CQ was harmful to PMVECs under HSS-induced PH, another study showed that CQ was effective in prevention of monocrotaline-induced PH.[26] Since autophagy is a double-edged sword serving both as a self-salvation mechanism and a cell death mechanism, it is not surprising that it can play both good and evil roles in different stages and different types of PH. Given the complex nature of PH, it remains challenging to fully understand how to harness the autophagic activity in this disease.

In conclusion, our study suggested that EPC-CM could suppress autophagy in PMVECs under HSS-induced PH, and suppression of autophagy was toxic to PMVECs. This research provides some insights to understand the role of autophagy and EPCs in HSS-induced PH. Further research should be done to confirm the mechanisms by which autophagy or EPCs impact the initiation and progression of PH, and to develop therapeutics specifically targeting these pathways.

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Ethical approval: This study was approved by the institutional ethics committee of the hospital, and was carried out according to the Guide for Care and Use of Laboratory Animals.

Competing interest: None declared.

Contributors: XBJ and CJ contributed equally to this study. XBJ and CJ summarized the results and wrote the first draft of the paper. All authors contributed to the intellectual content and approved the final version. TLH and CX are the guarantors.

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