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Vps15 is critical to mediate autophagy in AngII treated HUVECs probably by PDK1/PKC signaling pathway

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ABSTRACT

Aims: Vps15 is an important regulator on the activity of class III PI3K in autophagy induction. AngII plays a positive role of autophagy in the early protection of endothelial cells. In this study, the expression of Vps15 was knocked down using the specific shRNA to investigate the effects of Vps15 on cell autophagy, senescence and apoptosis in HUVECs stimulated by AngII. The associated cell signaling pathway was also explored.

Materials and methods: MDC staining was applied to show autophagic bodies. Cell senescence was detected using β-galactosidase staining. Cell apoptosis was examined by flow cytometry using Annexin V-FITC/PI staining. And western blot was used to evaluate the ratio of LC3-II/I and the activation of associated cell signaling pathway.

Key findings: Cell autophagy induced by AngII was inhibited in HUVECs transfected with Vps15-shRNA, while cell senescence and apoptosis were enhanced. Rescue experiment revealed that cell autophagy was activated after Vps15 reexpression, while cell senescence and apoptosis were inhibited. Moreover, the phosphorylations of PDK1 and PKC substrates were increased after AngII treatment, which were decreased by Vps15 knockdown. Pretreatment of cells with the inhibitor for PDK1 or PKC attenuated cell autophagy after AngII stimulation, yet promoted cell senescence and apoptosis. The phosphorylations of both PDK1 and PKC were inhibited in cells pretreated with PDK1 inhibitor. Only the activation of PKC was inhibited when the inhibitor for pan-PKC was used.

Significance: These results suggested that Vps15 was critical to the protective autophagy in HUVECs induced by AngII, and PDK1/PKC signaling pathway was probably involved.

1. Introduction

It is an important scientific issue to understand the biological process of senescence and to improve the quality of life in the precaution and early treatment of human diseases correlated with senescence. Variable cytokines and neurohumoral factors are involved in the process of senescence, which is regulated by multiple signaling pathways. The senescence of endothelial cell is reported to be associated with many common cardiovascular diseases like atherosclerosis, hypertension and heart failure. The disfunction of endothelial cells caused by senescence is an initial or promoting factor during the incidence of cardiovascular diseases [1]. It is a hotspots in the world around to investigate the molecular mechanisms of senescence recently, and targeting the inhibition of endothelial cell senescence will facilitate to find something new in the prevention and therapy of cardiovascular diseases.

Some progress has been made in studies concerning autophagy and senescence recently, and a strong correlation was established between them. The decrease or disregulation of autophagic activity with aging significantly affected the process of senescence, especially in cardiovascular system [2]. The increase of autophagic activity is required for an extended lifespan of variable eukaryotes, which is a critical regulatory mechanism associated with senescence. It was reported that autophagy as an important quality control system could facilitate maintaining the cardiac homeostasis and the adaptation to stress response. When treated with rapamycin, a strong autophagic inducer, the heart function was protected and the lifespan was prolonged in mice [3]. Multiple signaling pathways affect the autophagy of cardiomyocyte induced by AngII and constitute complicated regulating networks with autophagic molecules [4–7]. It is probable that the autophagic activity is declined with aging, which results in the dysfunction of endothelial cells. The senescence of blood vessel could be retarded by some kind of medicine like resveratrol, by activating cell autophagy [8]. We have reported that the autophagy of endothelial cells stimulated by AngII

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probably interfered with cell senescence and apoptosis [9].

Three relatively independent processes are involved in autophagy, including autophagy induction, formation of autophagic bodies and degradation of mature bodies, which are precisely modulated by variable signaling molecules. In the stage of autophagy induction, class III PI3K is a critical regulatory factor and is composed of Vps34 (catalytic subunit) and Vps15 (regulatory subunit) [10]. Vps34 is interacted with the autophagic core protein of beclin-1, and other autophagy-related factors are recruited to initiate autophagy process [11]. Vps15 is a serine/threonine protein kinase and is very important to the stability and activity of Vps34. When coexpressed with Vps15, the activity of Vps34 is significantly enhanced [12].

Angiotensin II (AngII) is the main factor of cell senescence in cardiovascular system. The pathological changes induced by AngII are similar to that found in senescent blood vessels. Our previous research found a positive role of autophagy in the early protection of endothelial cells stimulated by Ang II. Studies of AngII induced cell autophagy are focused on the process of the formation of autophagic bodies, and more and more autophagy-related molecules are disclosed. In this study, the regulatory subunit of class III PI3K, Vps15 was knocked down to investigate the regulatory effects of Vps15 on AngII induced autophagy and senescence of vascular endothelial cells.

2. Materials and methods

2.1. HUVECs culture and transfection

HUVECs were preserved in the Science and Experiment Center of China Medical University, and cultured in RPMI1640 medium (Gibco, USA), supplemented with 10% fetal bovine serum (FBS), in incubator at 37 °C with 5%CO2. HUVECs (80–90% confluence) were transfected with plasmid containing shRNA specific for Vps15 and the scramble control (both from OBiO Technology, China) using Lipofectamin 3000 (Invitrogen, USA) to reduce the expression of Vps15. 24 h after transfection, cells were selected by G418 to obtain stable cell lines with decreased expression of Vps15. Ang II (10 μM) was applied to induce cell autophagy for 24 h. For reexpression of Vps15, the expression plasmid (OBiO Technology, China) was transiently transfected into the stable cells mentioned above. The inhibitor for PDK1 or PKC was applied to cells in advance, 24 h before AngII treatment. The experiments for cells were repeated at least three times.

2.2. MDC staining

The autophagic bodies were shown in cells using a MDC staining kit (KeyGEN BioTECH, China). The staining was performed according to the manufacturer's instructions. Briefly, cells were washed with wash buffer for twice and incubated with appropriate volume of MDC working solution for 30 min in the dark. Cells were immersed in collection buffer and observed under an inverted fluorescence microscope. The number of autophagic bodies was counted in 10 cells at 10 randomly selected fields of 400× to evaluate the autophagic level in cells of different groups. The MDC staining was repeated in three replicates.

2.3. Senescence-associated β-galactosidase (β-gal) staining

Cell senescence was evaluated using β-galactosidase staining kit (Beyotime, China). Cells cultured on 60 mm dish were washed with PBS and fixed with 2% formaldehyde/2% glutaraldehyde for 15 min. Cells were washed and incubated at 37 °C overnight with staining working solution, including β-galactosidase and the X-Gal substrate provided in the kit. Then the dish was sealed with parafilm to avoid evaporation. Senescent cells were identified as blue-stained under an inverted microscope and a minimum of 100 cells was counted in 5 randomly selected fields of 200× to calculate the percentage of β-gal-positive cells. Three independent experiments for β-gal staining were performed.

2.4. Annexin V-FITC/PI staining

Cell apoptosis was examined using the Annexin V-FITC/PI staining kit (KeyGEN BioTECH, China). Cells were digested using trypsin with EDTA-free, collected and washed by centrifugation, and resuspended in 500 μL binding buffer. Cells were mixed with 5 μL Annexin V-FITC and then incubated with 5 μL PI for 10 min at room temperature avoid of light. Cell apoptosis was detected in flow cytometry (FACSAria, BD). The tests were repeated for three times.

2.5. Western blot

Cells were washed with ice-cold PBS, and lysed on ice in RIPA lysis buffer containing the mixture of protease inhibitors (Promega, USA) and phosphatase inhibitors (Solarbio Life Sciences, China). The lysates were centrifuged at 15000 rpm for 30 min at 4 °C. The supernatant was preserved and protein concentration was determined by the BCA kit (Beyotime, China). 50 μg of total protein was separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. 5% bovine serum albumin (BSA) was used to block unspecific binding of antibodies. The primary antibody of LC3 (1:500), PDK1 (1:1000), PKC (1:1000) and antibodies (1:1000) for substrates of various signaling pathways in the KinomeView Profiling kit (all from Cell Signaling Technology) were incubated on the membranes overnight at 4 °C. The KinomeView Profiling Kit (Cell Signaling Technology, #9812, containing 16 Phospho-motif Antibodies) provides a set of Phospho-motif Antibodies that cover a large portion of the kinome and react broadly with serine, threonine, and tyrosine phosphorylation mediated by diverse kinase families throughout the kinome and helps to determine the kinase families involved in the regulation of diverse physiological processes. The membranes were then incubated with goat anti-mouse or -rabbit IgG (1:5000, ZSGB-Bio, China) for 2 h at room temperature. The protein strips were developed using the enhanced chemiluminescence (ECL) system (Millipore, USA). The DNR Imaging System was used to catch up the specific protein bands and the integrated optical density (IOD) was measured using the software of Image Pro-Plus 6.0. The ratio of $IOD_{target\ protein}/IOD_{\beta\text{-}actin}$ was calculated as relative content and expressed graphically. The western blot experiments were performed in triplicate.

2.6. Statistical analysis

The statistical analysis software of SPSS 13.0 was applied to perform data analysis. One-way ANOVA was used to compare the differences between groups with various treatments. All data were represented as mean ± SD and results were considered statistically significant when the *p*-value was less than 0.05.

3. Results

3.1. Vps15 knockdown attenuated AngII-induced cell autophagy

HUVECs were stably transfected with specific shRNA of Vps15 to explore the function of Vps15 on AngII-induced cell autophagy. The decreased expression of Vps15 was confirmed in transfected cells (Fig. 1A). The results showed that the ratio of LC3II/I in control and scrambled-shRNA groups were higher than that in Vps15-shRNA transfected group (Fig. 1A). The number of autophagic bodies represented by MDC-staining in cells of control and scrambled-shRNA transfection group was more than that in Vps15-shRNA transfected cells (Fig. 1B). It was indicated that cells with Vps15 knockdown showed a decreased autophagic activity compared with other groups. Our results indicated that suppression of Vps15 expression interfered with cell autophagy induced by AngII.

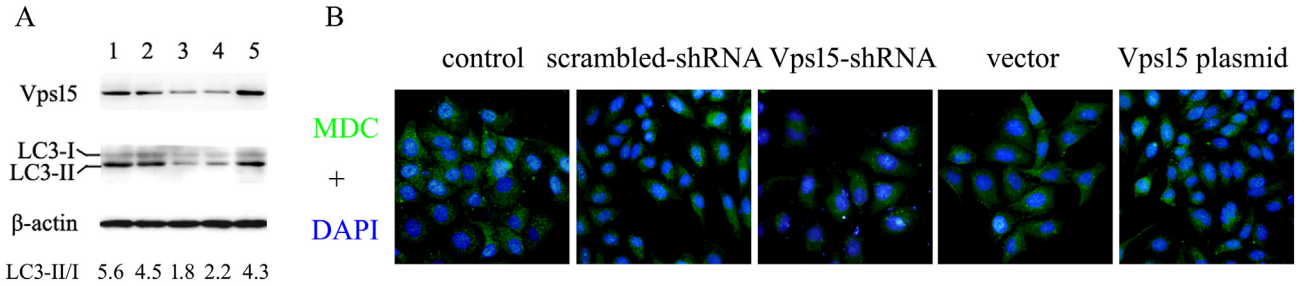

Fig. 1. A, Vps15 knockdown and reexpression was confirmed by western blot. Cell autophagy was detected by the ratio of LC3-II/I. Compared with control and scrambled-shRNA group, cells with Vps15 knockdown showed a decreased ratio of LC3-II/I, which was elevated by Vps15 reexpression. 1, control; 2, scramble shRNA; 3, Vps15-shRNA; 4, vector; 5, Vps15 plasmid. B, Cell autophagy was detected by MDC staining. Compared with control and scrambled-shRNA group, the number of autophagic bodies was less in Vps15-shRNA transfected cells, which was increased by Vps15 reexpression. (400×).

3.2. Vps15 knockdown enhanced cell senescence and apoptosis stimulated by AngII

β-Galactosidase and Annexin V-FITC/PI staining were applied in this study to investigate the effects of Vps15 knockdown on cell senescence and apoptosis stimulated by AngII. The β-galactosidase positive rates in cells of control, scrambled and Vps15-shRNA transfection groups were 22.4 ± 1.9%, 24.3 ± 2.1% and 42.5 ± 2.5%, respectively ($p = 0.000$, Fig. 2A). The apoptotic rates in cells of control, scrambled transfection groups were 11.9 ± 1.0% and 10.4 ± 1.1%. When transfected with Vps15-shRNA, the percentage of apoptotic cells was 24.6 ± 1.7%, which was apparently increased ($p = 0.000$; Fig. 2B). Our results showed that cell senescence and apoptosis were significantly enhanced in Vps15 shRNA-transfected cells.

3.3. The reexpression of Vps15 rescued the effects of its knockdown on AngII treated cells

Since Vps15 knockdown interfered with AngII initiated cell autophagy, the rescue experiment was performed to further improve our conclusions. Cells with Vps15 knockdown were transfected with Vps15 expression plasmid, and cell autophagy, senescence and apoptosis were examined. Our results showed that higher ratio of LC3II/I (Fig. 1A) and more autophagic bodies (Fig. 1B) were found in cells with reexpression of Vps15. Moreover, both the β-galactosidase positive- (23.1 ± 2.7%) and apoptotic rates (10.4 ± 1.1%) in cells with Vps15 reexpression were lower than in cells of Vps15 knockdown group ($p = 0.000$, Fig. 2A and $p = 0.000$, Fig. 2B).

3.4. The PDK1/PKC signaling pathway was involved in the Vps15 regulated cell autophagy induced by AngII

In this study, the KinomeView Profiling Kit was used to explore the probable signaling pathway(s) involved in Vps15 mediated cell autophagy in HUVECs stimulated by AngII. Significant differences between each group were found when the antibodies of phospho-(Ser/Thr) PDK1 docking motif (18A2) mouse mAb and phospho-(Ser) PKC substrate (P-S3-101) rabbit mAb in the kit were used. The results showed that the phosphorylation of PDK1 substrates was increased after AngII treatment, which was interfered by Vps15 knockdown (Fig. 3A). We also found that the phosphorylation level of PKC substrates was elevated by AngII stimulation, which was decreased when Vps15 was down-regulated (Fig. 3A). To further investigate whether the activated PDK1/PKC signaling pathway was definitely involved in Vps15 mediated cell autophagy in HUVECs induced by AngII, the phosphorylation antibody specific for PDK1 or PKC was applied. It was shown that the phosphorylation of PDK1 and PKC was activated in AngII treated HUVECs. However, when Vps15 was knocked down, the activation of PDK1 and

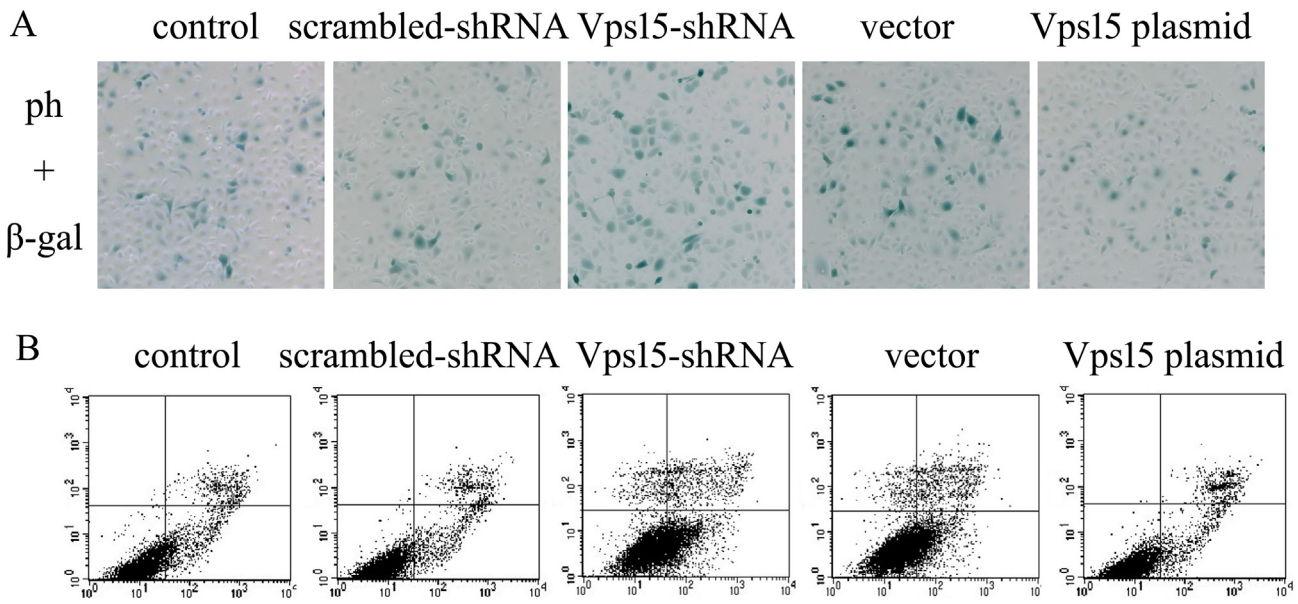

Fig. 2. A, Cell senescence was detected by β-galactosidase staining. Compared with control and scrambled-shRNA group, more cells with Vps15 knockdown showed β-galactosidase staining, which was decreased by Vps15 reexpression. (200×) B, Cell apoptosis was detected by Annexin V-FITC/PI staining. Compared with control and scrambled-shRNA group, the apoptotic rate in Vps15 knockdown cells was increased significantly, which was decreased by Vps15 reexpression.

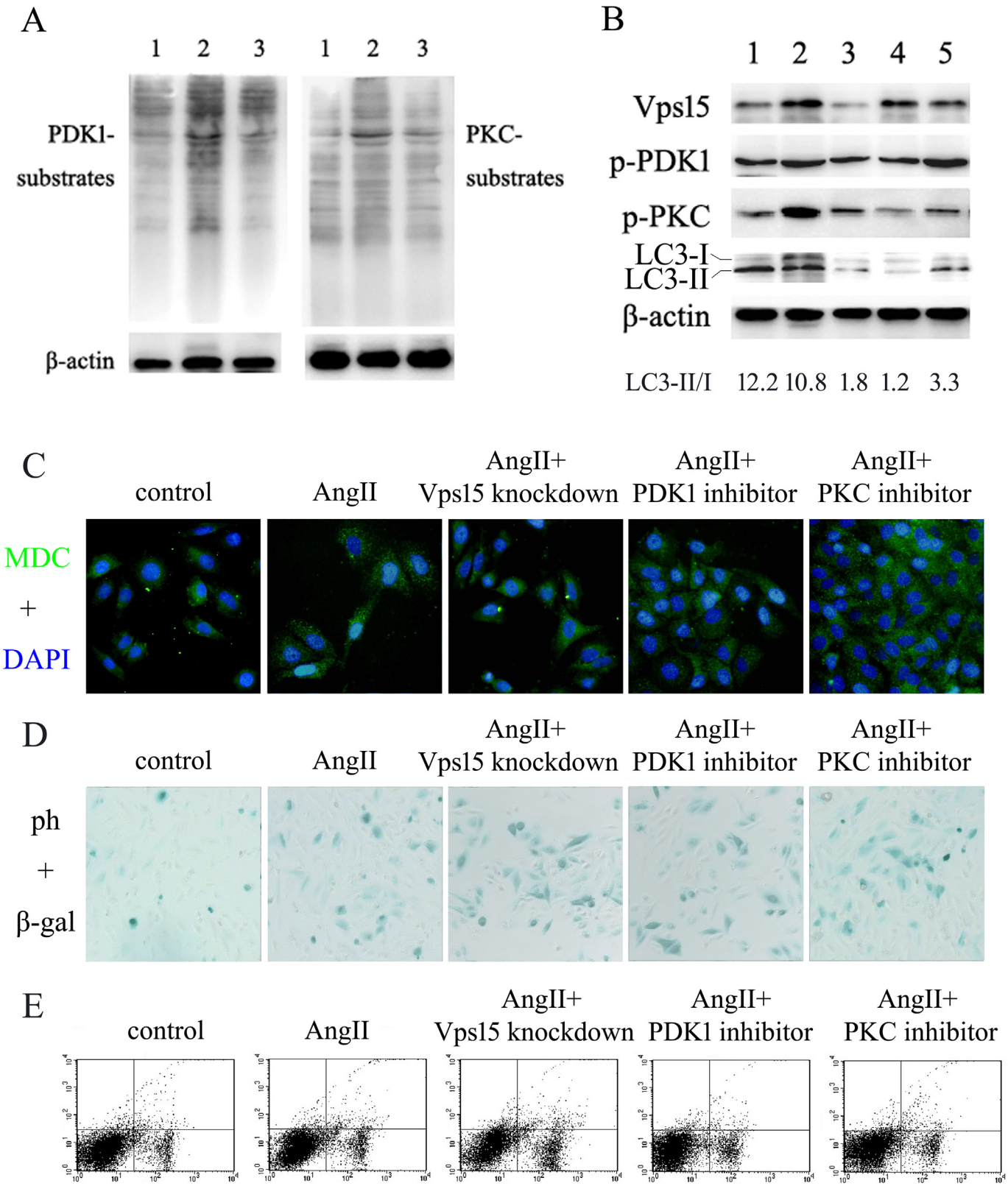

Fig. 3. A, The substrates phosphorylation of PDK1 and PKC were affected by Vps15 knockdown in HUVECs stimulated by AngII treatment. B, The phosphorylation of PDK1 and PKC were activated in AngII treated HUVECs. When Vps15 was knocked down, the activation of PDK1 and PKC was inhibited. When the inhibitor for PDK1 was used, both phosphorylation level of PDK1 and PKC was decreased. When the inhibitor for pan-PKC was used, only the activation of PKC was inhibited. C, The number of autophagic bodies was less in PDK1 or PKC inhibitor pretreated cells than AngII stimulated cells (400×). D, More cells pretreated with PDK1 or PKC inhibitor showed β-galactosidase staining, compared with AngII stimulation group (200×). E, The apoptotic rate in PDK1 or PKC inhibitor pretreated cells was increased significantly, in comparison to cells with AngII treatment alone. 1, control; 2, AngII treatment; 3, AngII + Vps15 knockdown; 4, AngII + PDK1 inhibitor; 5, AngII + PKC inhibitor.

PKC was inhibited. When the inhibitor for PDK1, GSK2334470 (MCE, USA) was used, the expression of Vps15 was unaffected, however both phosphorylation level of PDK1 and PKC was decreased (Fig. 3B). Moreover, when the inhibitor for pan-PKC, Go 6983 (MCE, USA) was used, no change was found concerning Vps15 expression and PDK1 phosphorylation, only the activation of PKC was inhibited (Fig. 3B).

The effects of PDK1 inhibitor or PKC inhibitor on Ang II induced autophagy, senescence and apoptosis were also investigated. In comparison to cells stimulated with AngII alone, we found a lower ratio of LC3-II/I (Fig. 3B) and less autophagic bodies in cells pretreated with PDK1 inhibitor (Fig. 3C). Moreover, both the β-galactosidase positive- (30.3 ± 1.6%) and apoptotic rates (18.6 ± 1.9%) were higher than cells with AngII treatment ($p = 0.000$ and $p = 0.007$, Fig. 3D and E). The similar results were also found in cells pretreated with PKC inhibitor. In comparison to cells treated with AngII alone, we found a lower ratio of LC3-II/I (Fig. 3B) and less autophagic bodies in cells pretreated with PKC inhibitor (Fig. 3C). Furthermore, both the β-galactosidase positive- (32.6 ± 2.0%) and apoptotic rates (17.3 ± 2.3%) were higher than cells with AngII stimulation ($p = 0.000$ and $p = 0.029$, Fig. 3D and E). These findings further revealed that AngII induced autophagy, senescence and apoptosis were associated with PDK1 and PKC signaling.

4. Discussion

Autophagy is some kind of cell protection to avoid cell death under nutrient deficiency or oxidative stress. Reduction of autophagy induces degenerative changes in mammalian cells that similar to those pathological status associated with aging. Studies have reported that AngII is involved in the senescence of endothelial cells and plays important roles in cardiovascular diseases [13]. We have previously confirmed that AngII induced autophagy, senescence and apoptosis of HUVECs progressively. Autophagy provided an early protection on endothelial damage due to Ang II stimulation [9]. Vps15 is the regulatory unit of class III PI3K, which is participated in the formation of associated protein complex critical for autophagy initiation [14,15]. The complete ablation of Vps15 resulted in the accumulation of autophagic substrates, the induction of apoptosis and severe cortical atrophy, which indicated the importance of the Vps15-Vps34 complex in the development of telencephalon [16].

In this study, Vps15 was knocked down in HUVECs to explore the indispensable role of Vps15 in the process of AngII induced cell autophagy. We found that HUVECs exhibited less expression of Vps15 after the transfection of specific shRNA, and a decreased autophagic activity induced by AngII was observed. Our results indicated that Ang II stimulated cell autophagy was interrupted by Vps15 knockdown. Moreover, once cell autophagy was interfered, more β-gal staining and apoptosis were found after AngII treatment, which suggested an effective contribution of Vps15 in preventing the adverse influence of Ang II on endothelial cells. Our conclusion was further confirmed by Vps15 rescue experiments. Vps15 was reexpressed in HUVECs with Vps15 knockdown and the autophagic activity was found increased, while cell senescence and apoptosis were inhibited under the stimulation of AngII. These results indicated that Vps15 probably mediated cell autophagy induced by AngII in HUVECs.

Although these results are only preliminary in nature, there are reasons to believe that Vps15 is like a switch between autophagy and senescence stimulated by AngII in endothelial cells. In AngII stimulated endothelial cells, the primary receptor of AT1 signaling would be activated to induce cell senescence; Class III PI3K (Vps34/Vps15) could be activated to form core complex of autophagy to initiate cell autophagy. Vps15 is supposed to be applied in the internalization of AT1 receptor to form endosome vesicles, which are transported to lysosomes for degradation, and interference with AT1 signaling to block cell senescence. Therefore, we presume that class III PI3K (especially Vps15) regulate the formation of autophagic complex, as well as the internalization and transportation of AT1 receptor, like a switch between autophagy and senescence.

The signaling pathways involved in the process of autophagy activation may vary in different cells under different physiological or pathological conditions [17]. Disturbance of RAF/MEK/ERK signaling elicited cell autophagy, which protected PDA cells from the cytotoxic effects of KRAS pathway inhibition [18]. In this study, the KinomeView Profiling Kit was used as a primary screening for the possible signaling pathway(s) participated in Vps15 mediated cell autophagy induced by AngII. We found a general decrease in the phosphorylation level of PDK1 or PKC substrates in Vps15 knockdown cells. To further validate our inference that PDK1/PKC signaling pathway was probably necessary for Vps15 mediated cell autophagy, the specific inhibitor for PDK1 or PKC was applied. The activation of PDK1 was observed after AngII treatment, which was reduced when Vps15 was downregulated. The phosphorylation levels of PDK1 and PKC were both decreased by the specific inhibitor of PDK1. The phosphorylation of PKC was interfered by the inhibitor for pan-PKC, while the activation of PDK1 was not affected. Our results suggested that the PDK1/PKC signaling pathway was involved in AngII induced cell autophagy, which was definitely mediated by Vps15. Our findings may provide a new insight in the molecular mechanism associated with AngII induced cell autophagy. PDK1 was confirmed to be a strong potential regulator in osteoclast stimulatory pathways (Akt, ERK) and autophagy induction (via mTORC1), probably contributed to the osteoclast phenotype in Paget's disease of bone [19]. PDK1 was also shown to mediate autophagy regulated by miRNAs. MiR-155-5p promoted autophagy of cervical cancer cells. Whereas miR-155-5p downregulation suppressed cell autophagy through promoting the PDK1/mTOR pathway [20]. MiR-378 promoted cell autophagy through the mTOR/ULK1 pathway and sustained autophagy via FoxO-mediated transcriptional enhancement by targeting PDK1 [21]. Studies have focused on the connection of PKC and autophagy, and it is inferred that different duration of autophagy stimulation and distinct cell contexts result in various function of PKC in regulating cell autophagy [22]. Irbesartan, an inhibitor of AngII receptor, induced cell autophagy, inhibited PKC and activated AMPK and its downstream effector ULK1, thereby decreasing lipid deposition and restoring mitochondrial function in the steatotic hepatocytes stimulated by AngII [23]. Yet, how Vps15 influences PDK1/PKC signaling pathway in the autophagy initiation needs further investigations.

In summary, the data presented in this study demonstrated that Vps15 was considerably critical for AngII-induced endothelial cell autophagy, possibly via the activation of PDK1/PKC signaling pathway. Moreover, a better understanding of the mechanism by which Vps15 regulates cell autophagy induced by AngII would be beneficial to the development of new therapeutics for aging-associated cardiovascular diseases.

Declaration of Competing Interest

The authors declare they have no competing interests.

Acknowledgments

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