PEDF Induces Native Coronary Collateral Microcirculation Remodeling in a Manner Similar to That of FSS Through Activing Canonical and Non-canonical Notch1 Signaling Pathway Simultaneously

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Original investigation

Keywords: PEDF, CCMR, native collaterals, Remodeling, Notch1 signaling pathway

Posted Date: April 3rd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-19733/v1

Abstract

Background Our previous studies showed that the coronary collateral microcirculation reserve (CCMR) in rat hearts is abundant, but the structure is naturally flawed, which preventing it from continuously providing alternative blood flow to the ischemic myocardium. Further research indicated that pigment epithelium-derived factor (PEDF) can induce CCMR vessels remodeling in a manner similar to that of fluid shear stress (FSS), thus improving compensatory blood flow. However, the specific mechanism remained unclear. Methods We established the rat model of PEDF overexpression in the myocardium and the cardiac explant angiogenesis model in matrigel to identify the role of the canonical and non-canonical Notch1 signaling pathway in the process of PEDF-induced CCMR remodeling. Results: We found that pharmaceutical blockage of Notch1 pathway via γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) would effectively abrogate the remodeling process, including the diameter expansion and adherens junctions reorganization of CCMR induced by PEDF. In vitro, western blot and enzyme activity assay analysis indicated that PEDF treatment simultaneously active the canonical Notch1 pathway (NICD/AKT/eNOS/NO) and non-canonical Notch1 pathway (TMD/VE-cadherin-LAR-Trio complex). To our amusement, using L-Nitromonomethylarginine (L-NMMA) acetate to inhibit endothelial nitric oxide synthase (eNOS) activity could significantly block PEDF-induced the diameter expansion of the nascent blood vessels, but it had little effect on the reassembly of adherens junctions. While activation of non-canonical Notch1 pathway seems to be the cause of adherens junctions remodeling. Blocking of the non-canonical Notch1 pathway canceled PEDF-driven adherens junctions assembly. Conclusions We demonstrate the specific mechanism of PEDF-induced native collateral microcirculation remodeling. PEDF can active the canonical Notch1 pathway signaling pathway to promote lumenal remodeling and, Simultaneously, active non-canonical Notch1 signaling pathway responsible for adherens junctions assembly.

Background

During acute myocardial infarction (AMI), native coronary collateral circulation provides an alternative source of blood supply for ischemic myocardium in time[1-3]. The extent of the native coronary collateral circulation (ie, preexisting collateral number and diameter) is a primary determinant of the severity of myocardial infarction following acute coronary obstruction. Additionally, the function of the coronary collateral circulation has a great impact on the prognosis of AMI. A well-developed coronary collateral circulation is associated with improvement of cardiac function and reduction of future adverse cardiovascular events[4, 5].

Our previous study demonstrated that coronary collateral microcirculation reserve (CCMR) is an important component of the coronary collateral circulation system in rats. During AMI, a large number of CCMR vessels were recruited to regulate coronary blood flow and myocardium perfusion. However, our further research showed that CCMR vessels of normal adult rats have natural defects in structure, as indicated by discontinuous basement membranes, less vascular endothelial cadherin (VE-cadherin) assembly and

greater permeability[6]. VE-cadherin, one of the proteins forming the adherens junctions, plays an important role in maintaining vascular integrity and stability[7-9].

Pigment epithelium-derived factor (PEDF), a 50-kDa secreted glycoprotein, is widely expressed throughout the body. Previous studies have shown its role in maintaining homeostasis, including neuroprotective, anti-oxidative, anti-inflammatory, anti-tumor and anti-angiogenic[10-13]. It was found in our previous research that PEDF induced CCMR vessels remodeling-- a process known as “pruning”, as manifested by increases in lumen diameter and the assembly of VE-cadherin. Briefly, on the one hand, the number of CCMR vessels was reduced, and the diameter increased. Ultimately, the total cross-sectional area of CCMR vessels increased, resulting in an increase in the total blood flow perfusion for the myocardium. On the other hand, the assembly of VE-cadherin was significantly improved, resulting from the transfer of VE-cadherin from cytoplasm to membrane. Leakage from CCMR was reduced, and the opening time of CCMR was prolonged after AMI. We previously speculated that PEDF induced CCMR remodeling in a manner similar to the way fluid shear stress (FSS) works and that may be associated with the Notch1 or nitric oxide (NO) pathway[14]. However, there is no sufficient evidence to support it, and its specific mechanism is unknown.

Previous studies showed a connection between FSS and the Notch1 signaling pathway. FSS regulates adherens junctions and vascular barrier function through the Notch1 signaling pathway[15, 16]. Notch1 signaling pathway regulating vascular proliferation and differentiation, vascular barrier function and the formation native collateral networks is vital in the cardiovascular system[17-20]. In mammals, ligands of the Delta-like 4 (Dll4) expressed on a neighboring cell combined with Notch1 receptors, triggering a cascade of enzymatic cleavages of the receptor by ADAM family members and the γ-secretase complex. This causes the release of the Notch1 intracellular domain (NICD) which mediates the canonical Notch1 signaling pathway and reveals the Notch1 transmembrane domain (TMD)—the key domain that mediates the non-canonical Notch1 signaling pathway. NICD participates in many signaling pathways responsible for aspects of cell life activity [21-23]. Polacheck et al reported that TMD catalyze the formation of a novel receptor complex in the plasma membrane consisting of VE-cadherin, the transmembrane protein tyrosine phosphatase LAR, and the Rac1 GEF Trio. This complex activates Rac1 to promote adherens junctions assembly and establish barrier function[16].

NO, an important vasodilation factor, is the major mediator in controlling vascular remodeling. Studies showed that the NO signaling pathway is also associated with FSS-induced lumenal remodeling[24, 25].

We previously proposed and preliminarily verified the hypothesis that PEDF induces CCMR remodeling in a similar manner that FSS does. Interestingly, our previous research also showed that the addition of exogenous PEDF to endothelial cells significantly increased the expression of NICD and endothelial nitric oxide synthase gene (eNOS), and activated Notch1 and NO signaling pathways under normoxic condition[14]. Therefore, we speculate that PEDF-induced CCMR remodeling may be related to the Notch1 and NO pathways. The purpose of this study is to explore the specific mechanism in which PEDF induces vascular remodeling.

Methods

The present study was performed conforming to the National Institutes of Health (NIH Publication, 8th Edition, 2011) guidelines in the use of laboratory animals. The animal care and experimental protocols were approved by the Xuzhou Medical University Committee on Animal Care.

Preparations of Lentivirus (LVs) and Plasmids

Recombinant lentivirus (PEDF-LV; Shanghai GeneChem Co,.Ltd, Shanghai, China) was prepared as previously described[26]. eNOS overexpression plasmids was successfully constructed and then packaged in 293T cells. The concentrated titer of virus suspension was 2×10^{12} TU/L.

Animal Feeding and Treatment

Sprague-Dawley male rats (weighing 250 ± 10 g, at 8–10 weeks of age, n=30) were obtained from the Experimental Animal Center of Xuzhou Medical College. Rats were housed in a temperature-controlled environment (22°C) with a 12-hour light/dark cycle. Rats had free access to food and water.

The γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) was obtained from MedChem Express (MCE, USA).All rats were randomly divided into 5 groups: the control group, vector group, PEDF-LV group, PEDF-LV + DMSO group, and PEDF-LV + DAPT group. The rats of the control group were only ligated with left anterior descending coronary artery (LAD). Lentivirus vector in 20 μl enhanced infection solution was delivered with a 20-μl syringe and 25-gauge needle into the myocardium along the LAD coronary artery in rats of the vector group. PEDF-LV (2×10^7 TU) in 20 μl enhanced infection solution was delivered into the myocardium along the LAD coronary artery in rats of the PEDF-LV group, PEDF-LV + DMSO group, and PEDF-LV + DAPT group. After 3 days, the rats of the PEDF-LV + DMSO group and PEDF-LV + DAPT group were injected intraperitoneally with DMSO or DAPT (100 mg/kg body weight) every 2 days (total of 5 injections).

Rat AMI Model

The AMI model was established surgically by ligation of the left anterior descending (LAD) coronary artery in anesthetized rats as [27]. Briefly, SD rats were anesthetized by intraperitoneal injection of 60 mg/kg sodium pentobarbital maintained under anesthesia using isoflurane (1.5–2.0%) mixed with air. Rats were placed on the operating table in a supine position and performed left thoracotomy through the fourth intercostal space under sterile condition. Then intramyocardial gene delivery was performed through myocardium injection directly. After reinstallation of spontaneous respiration, animals were extubated and allowed to recover from anesthesia, and postoperative analgesia was performed with buprenorphine administration at 0.5 mg/kg. Rats had the standard diet after surgery. 7 days later, we exposed the heart of rats and then occluded LAD using 6-0 silk suture (Ethicon, Johnson & Johnson, New Brunswick, USA) to generate the AMI with the same surgery. 5 minutes after ligation, the rat hearts were removed under anesthesia.

Lectin-FITC perfusion experiment

Lectin from Bandeiraea simplicifolia/ FITC (catalog No. L2895; Sigma-Aldrich, St. Louis, MO) non-specifically binds glycoproteins on the surface of endothelial cells and marks them in real time. We ligated the LAD of rats and injected one milliliter of Lectin-FITC (50 ug/ml) into their femoral veins. Lectin-FITC following the bloodstream to reach various grades of blood vessels marked CCMR vessels in the infarct area. After 5min of ligation, the hearts were harvested immediately to make frozen sections.

Vascular density measurement

Frozen sections from rat hearts that had been injected by lectin-FITC were fixed in 4% paraformaldehyde for 15 minutes, washed three times with PBS, and finally covered with 50% glycerol. Panoramas were acquired by scanning these samples using the Slide Scanning System (Olympus VS120). Ten randomly fields of view (15X) in the infarct area of each sample were selected to measure vessel density. Computer-based quantification of pictures was done with ImagePro Plus software (Media Cybernetics, Rockville, MD).

Preparations of PEDF Protein

Recombinant rat PEDF (GenBankTM Accession Number NM_177927) was synthesized by Cusabio Biotech, Co., Ltd. (Wuhan, China) as previously described [].

Cardiac Explant Angiogenesis Model

In order to explore the relationship between PEDF induced vascular remodeling and the Notch1 signaling pathway in vitro, we established the cardiac explant angiogenesis model as previously described[6, 28]. Put simply, we cut and rinsed the cardiac explants (1-2mm^3) from neonatal Sprague-Dawley rats (1–3 days old, weighing 6.00.5 g). The cardiac explants were seeded on 24-well tissue culture plates (Corning, USA) coated with 250 μl Matrigel Basement Membrane Matrix and cultured in ECM at 37°C. Since PEDF has a strong inhibitory effect on angiogenesis, we added PEDF or DAPT on day 3 following implantation and observed on day 6. The models were randomly divided into four groups as follows: i) Control group; ii) PEDF group, PEDF protein (10 nmol/ml) was added to ECM on day 3; iii) PEDF +DMSO group, PEDF protein (10 nmol/ml) and DMSO (1:1000) were added to ECM on day 3; iv) PEDF+DAPT group, PEDF protein (10 nmol/ml) and DAPT (50μM,) were added to ECM on day 3.(Figure 2A)

Cell Culture

Human coronary artery endothelial cells (HCAECs, ScienCell, catalog #6020) were maintained in ECM growth media (ECM, ScienCell, catalog#1001) and used at passages 3-6. Cells were maintained at 37 °C in 5% CO2 in a humidified incubator. The cell cultures were fed every second day. HCAECs were subcultured or subjected to experimental procedures at 80% to 90% confluence.

Detection of γ-Secretase Activity

The assay was carried out in a microplate reader (Synergy2, BioTek, USA) using a γ-secretase Activity kit according to the manufacturer's instructions. The γ-secretase activity kit (GMS50607.1 v.A) was obtained from GENMED SCIENTIFICS INC. USA. The peptide substrate double-labeled by fluorescent probe NMA donor and DNP acceptor were cleavaged by the γ-secretase, and released NMA with strong fluorescent signal. The principle of the assay is that the cell lysate is tested for secretase activity by detecting of the fluorescent signal intensity. The level of secretase enzymatic activity in the cell lysate is proportional to the fluorometric reaction.

Evaluation of NO production in fixed cells

Production of NO in HCAECs was assessed using NO indicator 3-Amino, 4-aminomethyl-2', 7'-difluorescein, diacetate (DAF-FM DA) assay kit purchased from Beyotime (Jiangsu, China). DAF-FM DA can pass through the cell membrane and be catalyzed by the intracellular ester enzyme to release DAF-FM, which reacted with NO to produce strong fluorescent signal at an emission wavelength of 515 nm and an excitation wavelength of 495 nm. The cells were detached from the culture dishes by trypsin treatment, followed by being loaded with DAF-FM DA (5 M) in PBS (pH 7.4) for 20 min at 37 ◦C. Thereafter, cells were washed thrice and resuspended in PBS. Fluorescence was detected with a microplate reader (Synergy2, BioTek, USA). The fluorometric reaction index the NO level.

Immunofluorescence

Sections, cells or explants were fixed, respectively, with 4% paraformaldehyde for 15 minutes, permeabilized with 0.1% Triton X-100, and blocked with solution containing 5% bovine serum.

Specimens were incubated with anti-CD31(catalog no. ab24590; 1:200; Abcam, Cambridge, MA) and anti-VE-cadherin (catalog no. ab33168;1:300; Abcam) together for12 hours in 4°C. Next, secondary antibodies (catalog no. A21207; 1:200; Life Technologies, Carlsbad, CA) and Alexa Fluor 488 goat antimouse immunoglobulin G (catalog no.A11001; Life Technologies) were applied subsequently under light-protected conditions for 1 hour at room temperature. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; KeyGen Biotech, catalog #KGA215–10, China). Specimens were rinsed three times over 15min with PBS between each treatment. Finally, the specimens were observed under a fluorescence microscope (Olympus) or confocal laser scanning microscope (Olympus).

Western Blotting

Myocardial tissue protein from the infarcted area in rats and the cell protein from HCAECs were extracted with lysis buffer (pH 7.5) containing protease and phosphatase inhibitors (Sangon Biotech, catalog#C510003). In the same way, the endothelial cell protein of cardiac explants Angiogenesis was also extracted. The only difference was that we need to pick the endothelial cells under the microscope before the above procedures.

Primary antibodies for VE-cadherin, CD31, Dll4 (G-12, 1:1000, Santa Cruz Biotechnology), Notch1 (A7636, 1:1000, ABclonal), Cleaved Notch1 (Val1744) (4147S, 1:1000, Cell Signaling Technology), eNOS (32027S,

1:1000, Cell Signaling Technology), phospho-endothelial nitric oxide synthase (P-eNOS) (9571S, 1:1000, Cell Signaling Technology), Akt (#3272S, 1:1000, Cell Signaling Technology), P-Akt (#9271S, 1:1000, Cell Signaling Technology), Trio (H-120, 1:1000, Santa Cruz Biotechnology), presenilin-1(PS1) (16163-1-AP, 1:1000, ProteinTech Group), β-actin (66009-1-AP, 1:1000, ProteinTech Group), LaminA/C (10298-1-AP, 1:1000, ProteinTech Group) or ATP1A1 (14418-1-AP, 1:1000, ProteinTech Group) were followed by fluorescently labeled antimouse or -rabbit antibodies (LI-COR Biotechnology, Lincoln, NE) and imaged using the Odyssey infrared imaging system (LI-COR Biotechnology). Quantification was performed using ImageJ.

Co-immunoprecipitation

After protein extraction with special lysis buffer, protein concentrations were measured by BCA assay. A total of 500 μg of the cell lysates was incubated at 4°C by gently rocking with 5 μg of anti-Notch1 or anti-Trio antibody for 12 hours. Then protein A/G agarose beads (catalog #9863, CST, USA) was added to bind with the complex for 2 hours with gently shake at 4°C. Co-immunoprecipitates were then magnetically separated, washed 3 times in lysis buffer, and finally revealed by Western blot analysis. Rabbit normal IgG (Santa Cruz) served as negative control.

RT-qPCR

Total RNA was extracted from endothelial cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Complementary DNA was synthesized using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Afterwards, 20-uL reactions with primers (GENEWIZ Inc., South Plainfield, USA) were detected by Light Cycler 480II (Roche, Basel, Switzerland) using SYBR Green PCR Master Mix (Applied Biosystems, Waltham, USA). The mRNA expression levels were calculated using $2-\Delta\Delta CT$ methods. The expression levels of PS1 was normalized to the expression of GAPDH.

The primers were as follows: PS1, forward, 5'- CATTATCTAATGGACGACCCCA -3' and reverse, 5'-AATGGGGTATAGATTAGCTGCC -3'; GAPDH, forward, 5'-CGAGATCCCTCCAAAATCAA -3' and reverse, 5'-TGTGGTCATGAGTCCTTCCA -3'.

Statistical Analysis

Data are expressed as the mean±SEM. Statistical analyses were performed using GraphPad Prism 7.0 or Microsoft Excel. Two independent sample data sets were tested using 2-tailed Student t test. Multiple comparisons utilized one-way ANOVA followed by Student-Newman-Keuls test. $P<0.05$ was considered to be statistically significant.

Results

1. Pharmaceutical blockade of Notch1 signaling using DAPT significantly inhibited the PEDF-induced CCMR remodeling in vivo

In order to examine the relationship between PEDF-induced CCMR remodeling and Notch1 signaling pathway, we applied DAPT to block the activation of Notch1 signaling pathway and observed the distribution and morphological characteristics of CCMR vessels in rat hearts (Figure 1A). Consistent with previous results, we found that PEDF treatment can significantly reduce the number of CCMR vessels in rat hearts (451.7±41.94 versus 1006±67.87 [control], P<0.001), but lead to the diameter expansion of the remaining collateral vessels (11.78±0.51 versus 7.69±0.41 [control], P<0.001), and we called this process “remodeling”. While DAPT treatment significantly blocked this remodeling process, including inhibition of the increase in lumen diameter (9.39±0.55 versus 11.78±0.51 [PEDF], P<0.01) and total cross-sectional area of CCMR vessels (23.28±0.29% versus 27.88±0.30% [PEDF], P<0.001) (Figure 1B-D). Similarly, immunofluorescence analysis showed that the functions of PEDF to reassemble loosely distributed VE-cadherin and remodel the endothelium adherens junctions were also partially abolished by DAPT (Figure 1E). These above findings demonstrated that the Notch1 pathway does participate in the process of PEDF-induced CCMR remolding.

2. DAPT treatment significantly reversed the remodeling process of nascent blood vessels caused by PEDF in vitro

To explore the precise mechanism of PEDF remodeling vessels, we selected the cardiac explant angiogenesis model in Matrigel for further research. As elucidated in previous study, PEDF can remodel the nascent blood vessels. We found that PEDF treatment effectively promoted selected vessels to be thicker and stronger (15.10±0.80 versus 7.55±0.69 [control], P<0.001). It is important to point out that these effects were inhibited by DAPT treatment as evidenced by suppression of diameter expansion (12.00±0.66 versus 15.10±0.80 [PEDF], P<0.05) (Figure 2B-D). Likewise, immunofluorescence analysis of cardiac explants demonstrated that DAPT treatment also remarkedly attenuated the reassembly of VE-cadherin caused by PEDF in endothelial cells (Figure 2E). This, together with the above findings, both in vitro and in vivo, indicated that the Notch1 pathway is involved in the process of PEDF-induced vascular remolding.

3. PEDF induces lumenal remodeling of nascent blood vessels via NICD/AKT/eNOS/NO (the canonical Notch1 pathway) signaling pathway

To observe the effect of PEDF on the Notch1 signal pathway, we detected the expression of proteins related to Notch1 signaling pathway in myocardium tissue and endothelial cells by western bolt. Results showed that PEDF treatment significantly increased the expression of Dll4 and NICD in myocardial tissue (Dll4: 0.56±0.03 versus 0.20±0.01 [control], P<0.001) (NICD: 0.58±0.03 versus 0.35±0.03 [control], P<0.001) and endothelium cells (Dll4: 0.59±0.03 versus 0.20±0.02 [control], P<0.001) (NICD: 0.60±0.02 versus 0.27±0.02 [control], P<0.001) compared with the control groups (Figure 3A). The key to activation of the Notch1 signaling pathway is the γ-secretase activity[29]. We found that PEDF treatment significantly increased γ-secretase activity in normoxic endothelial cells using the γ-secretase activity kit (12.26±0.53 versus 8.16±0.61 [control], P<0.01) (Figure 3B). It could be seen that PEDF significantly enhanced the activation of the Notch1 signaling pathway.

Our previous study showed that L-Nitromonomethylarginine (NMMA) acetate, a nitric oxide synthase inhibitor, also remarkably blocked the activity of PEDF-induced lumenal remodeling of nascent blood vessels. Based on the above results, we reasonably speculated that NO was involved in the process of PEDF activating the Notch1 signaling pathway to increase lumen diameter.

We examined the effect of PEDF on the production of NO using NO DAF-FM DA assay kit in endothelial cells. Results showed that PEDF significantly increased the production of NO which is mainly regulated by the activity of eNOS, especially its phosphorylation level (274.60±14.71 versus 132.00±7.58 [control], P 0.001) (Figure 3C). Previous studies have shown that NICD/Akt/eNOS is a common signal pathway that regulates the production of NO[30, 31]. Accordingly, western blot was used to detect the expressions of related proteins. The result showed that PEDF remarkedly increased the expressions of NICD, P-Akt, eNOS, and P-eNOS (NICD: 0.56±0.02 versus 0.31±0.02 [control], P 0.001) (P-Akt: 0.52±0.02 versus 0.31±0.03 [control], P 0.001) (eNOS: 0.88±0.03 versus 0.59±0.03 [control], P 0.001) (P-eNOS: 0.26±0.02 versus 0.11±0.01 [control], P 0.001). Surprisingly, DAPT treatment attenuated the increase of NICD, P-Akt, and P-eNOS caused by PEDF, but did not blunt the increase of eNOS (Figure 3D). In addition, the ratio of P-eNOS to eNOS in the PEDF + DAPT group was higher than that in the PEDF group (Figure 3E). This indicated that PEDF regulated the production of P-eNOS through NICD/Akt/eNOS, and PEDF might also increase the production of P-eNOS by increasing the expression of eNOS under normoxic conditions. To this end, we detected the expression of P-eNOS under different treatments. Results showed that production of P-eNOS also increased due to over-expressing eNOS but less than that under PEDF treatment (0.20±0.01 [eNOS-LV] versus 0.10±0.01 [control], P 0.01) (0.32±0.01 [PEDF] versus 0.20±0.01 [eNOS-LV], P 0.01) (Figure 3F). These findings suggested that PEDF regulated the production of P-eNOS not only through NICD/Akt/eNOS signaling but also through simply increasing the expression of eNOS.

In order to explore the role of the NICD/Akt/eNOS/NO signaling pathway in PEDF-induced vascular remodeling, we observed the number and morphological characteristics of nascent blood vessels in cardiac explants treated with PEDF or L-Nitromonomethylarginine (L-NMMA) (Figure 3G-I). As indicated in figure 3G, L-NMMA treatment interdicted PEDF-driven the diameter expansion of nascent blood vessels (11.25±0.68 versus 15.22±0.72 [PEDF], P 0.01) . Interestingly, immunofluorescence analysis showed that the reassembly of VE-cadherin caused by PEDF exhibited no change in in cardiac explants exposed to L-NMMA (Figure 3I). In addition, western blot analysis also showed that L-NMMA had little effect on the PEDF-induced redistribution of VE-cadherin (Mem: 0.50±0.01 versus 0.51±0.01 [PEDF], P>0.05) (Cyt: 0.21±0.01 versus 0.20±0.01 [PEDF], P>0.05) (Figure 3J). These results indicated that PEDF increased the lumen diameter via the canonical Notch1 pathway. However, PEDF-promoted the organization of adherens junctions might be mediated by other signaling pathways related to Notch1.

4. PEDF promotes assembly of adherens junctions in nascent blood vessels through non-canonical Notch1 signaling pathway

In a recent study (Nature 11/2017), Polacheck et al demonstrated that FSS regulated adherens junctions and vascular barrier function through a non-canonical Notch1 signaling pathway[16]. Our previous

research showed that PEDF enhanced the activation of Notch1 signaling pathway. Therefore, we reasonably speculated that PEDF drove adherens junctions assembly through the non-canonical Notch1 signaling pathway.

By co-immunoprecipitation assay, it was found that PEDF significantly promoted the formation of VE-cadherin/LAR/ Trio complex, but the effect of PEDF on the complex was inhibited by DAPT(Figure 4A). These indicated that PEDF can activate the non-canonical Notch1 signaling pathway.

NSC 23766 is a specific inhibitor of the binding and activation of Rac GTPase. As seen in Figure 4B, the expression of VE-cadherin on the membrane was reduced, and its intracellular expression increased in the PEDF+NSC group compared with the PEDF group (Mem: 0.33±0.01 versus 0.50±0.01 [PEDF], P□0.001) (Cyt: 0.42±0.01 versus 0.20±0.01 [PEDF], P□0.001). In other words, NSC almost completely prevented PEDF from driving VE-cadherin to be regular, tight, and orderly.

5. PEDF increases the expression of Dll4 protein and γ-secretase activity in endothelial cells

To investigate the precise mechanism of PEDF activating Notch1 signaling pathway, we examined the expression of proteins related to Notch1 signaling pathway. Western blot analysis showed that PEDF significantly increased the expression of Dll4. Moreover, co-immunoprecipitation assay showed that the binding of Notch1 and Dll4 increased in the PEDF group compared with the control group (Figure 5A and B). These findings indicated that PEDF increased Dll4 expression and promoted the binding of Notch1 receptor and Dll4 ligand to activate Notch1 signaling pathway.

Cleavages of the receptors by γ-secretase is an important node in the activation of Notch1 signaling pathway. PS1 is the most important catalytic subunit of γ-secretase complex. We found that PEDF significantly upregulated γ-secretase activity in endothelial cells (12.68±0.71 versus 8.15±0.54 [control], P □0.01) (Figure 5C). Next, we further observed the changes in PS1. Compared with the control group, the expression of PS1 total protein did not change significantly, the PS1 protein in the cytoplasm decreased while the PS1 protein on the membrane increased in the PEDF group (Total: 0.71±0.02 versus 0.70±0.02 [control], P□0.001) (Mem: 0.70±0.01 versus 0.50±0.02 [control], P□0.001) (Cyt: 0.28±0.01 versus 0.48±0.02 [control], P□0.001) (Figure 5D). Furthermore, reverse transcription-PCR showed that PEDF had no effect on PS1 mRNA levels (Figure 5E). These results indicated that PEDF promoted the transfer of PS1 from the cytoplasm to the membrane, and catalyzed γ-secretase activity of cleaving Notch1 receptor to activate the Notch1 signaling pathway.

Discussion

In this study, we concluded that PEDF remodels CCMR vessels in two main ways: (1) inducing dilation of the vascular diameter; (2) promoting endothelial adherens junctions assembly. Mechanically, PEDF triggers canonical Notch1 and non-canonical Notch1 pathways, which are respectively responsible for promoting the dilation of the vascular diameter and the regular, tight, and orderly assembly of VE-cadherin.

In previous research, the definition of "vascular remodeling" is confusing and has been used to designate various structural changes in different studies. Vascular remodeling, strictly speaking, is a structural change in the whole-vessel area circumscribed by the external elastic lamina (thus including the medial layer, internal elastic lamina, an eventual neointima, endothelial layer and vascular lumen). Here, PEDF-induced CCMR remodeling mainly exhibits the changes in the lumen diameter and vascuar barrier function. With respect to changes in the lumen diameter, remodeling can be outward or inward, leading to an increase or decrease in the total cross-sectional area of vessels[32]. FSS-induced remodeling is the clearest example of physiological remodeling, in which lumen caliber vary is directly proportional to changes in shear rates[33]. We found that changes in lumen diameter induced by PEDF were similar to outward remodeling induced by high FSS. The regulation of the vascular barrier function is also an important link in the process of vascular remodeling. Interestingly, we found that the role of PEDF in adherens junctions assembly under normoxic conditions is consistent with physiologically high FSS regulating endothelial barrier function. These indicate that the remodeling of both CCMR and nascent blood vessels driven by PEDF is similar to that caused by high FSS. Thus, we speculate that PEDF induces CCMR remodeling by activating some pathways regulated by FSS.

According to recent studies, possible pathways involved in the process are the Notch1 pathway, the NO pathway, the Rho pathway and the Ras-Raf-MEK-ERK pathway. However, our previous study showed that PEDF has little effect on the expression of proteins related to the Ras and Rho signaling pathway, but it significantly increased the expression of NICD and eNOS[14]. In addition, DAPT remarkedly blocks the remodeling effect of PEDF on vessels both in vivo and in vitro. These provide direct evidence that PEDF induces vascular remodeling through the Notch1 signaling pathway. Previous studies reported that the Dll4-Notch1 signaling pathway is involved in regulating the formation of native collateral networks and affects the extent of native collateral circulation[17]. This is consistent with the results of our study, that is, PEDF treatment significantly reduces the number of native collaterals and DAPT blocks this effect. However, the relationship between the decrease in the number of native collaterals induced by PEDF and the Dll4-Notch1 signaling pathway is still unclear. Additionally, Polacheck et al reported that the Dll4-Notch1 signaling pathway can regulate adherens junctions and vascular barrier function--a finding supporting our study that non-canonical Notch1 signaling pathway mediates the regulation in vascular barrier function caused by PEDF[16]. Importantly, Our study shows that PEDF can activate both canonical and non-canonical Notch1 signaling pathways to play a role in the process of PEDF-induced CCMR remodeling. PEDF can also regulate NO production to promote vascular outward remodeling via the canonical Notch1 signaling pathway.

NO is of great importance to the lumenal remodeling, and it is mainly regulated by P-eNOS[34]. We find that PEDF upregulates P-eNOS expression by the Notch1 signaling pathway and increasing eNOS expression. However, PEDF can significantly increase the ratio of P-eNOS to eNOS, while over-expression of eNOS cannot. In other words, PEDF not only increases the phosphorylation level of eNOS protein through the Notch1 signaling pathway, but also increases the basal level of eNOS protein to promote NO production, where the former plays a leading role. However, observing cardiac explants treated with L-

NMMA, we find that PEDF-upregulated NO through the canonical Notch1 signaling pathway is involved in the lumenal remodeling, but is not associated with the assembly of adherens junctions.

Here we show that PEDF induces an increase in lumen diameter via Notch1 / NO pathway. However, the specific mechanism responsible for NO regulating the vascular outward remodeling is poorly understood. Besides**,** more research on the receptors combined with PEDF in the process is needed.

Conclusions

In conclusion, our data reveal a novel mechanism that PEDF activates canonical and non-canonical Notch1 signaling pathway Simultaneously by increasing Dll4 expression and γ-secretase activity to promote lumenal remodeling and regulate vascular barrier function. This increases total cross-sectional area of CCMR vessels, improves the vascular barrier function and prolongs the time of native collateral vessels providing blood supply for ischemic myocardium to reduce myocardial injury and improve prognosis during AMI. This study provides a new experimental basis for a deeper understanding of the mechanism of PEDF-induced native coronary collateral remodeling, suggesting a new treatment strategy for fighting myocardial injury in AMI.

Abbreviations

CCMR: Coronary collateral microcirculation reserve; AMI: Acute myocardial infarction; eNOS: Endothelial nitric oxide synthase; VE-cadherin: Vascular endothelial cadherin; DAPT：N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; FSS: Fluid shear stress; L-NMMA: L-Nitromonomethylarginine; NO: Nitric oxide; Dll4: Delta-like ligand 4; NICD: Notch intracellular domain; TMD: Notch1 transmembrane domain; P-eNOS: Phospho-endothelial nitric oxide synthase; PS1: Presenilin-1;

Declarations

Ethics approval and consent to participate

The animal care and experimental protocols were approved by the Xuzhou Medical University Committee on Animal Care.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Nature Science Foundation of China (Grant No. 81570242 and 81270173) and Postgraduate Research & Practice Innovation Program of Jiangsu Province (Grant No. KYCX18_2190).

Authors' contributions

Jiali Chen and Xiucheng Liu contributed to experiment performance and drafted the manuscript. Xichun Qin, Hao Zhang, Zhiwei Liu, Lidong Zhu, Xiaoyu Quan, Yuzhuo Wang and Yeqing Zhou participated in data acquisition and analysis. Hongyan Dong and Zhongming Zhang contributed to the study design and critically revised the manuscript.

Acknowledgements

Not applicable.

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Figures

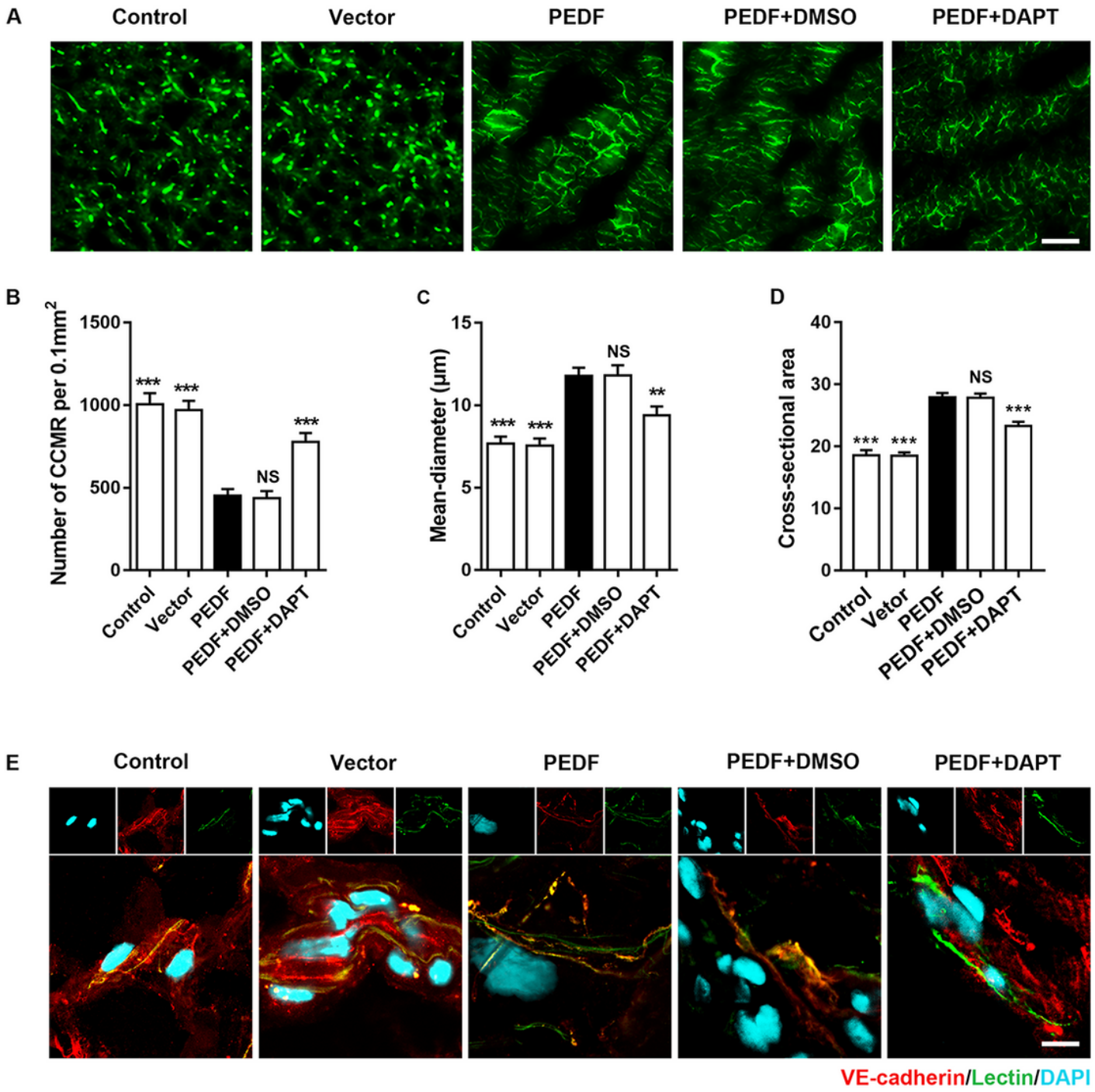

Figure 1

DAPT significantly inhibits PEDF-induced CCMR remodeling in vivo. A We carried out the lectin-FITC perfusion experiment in rats to mark CCMR vessels in infarction zone at 5 minutes ligation of the left anterior descending coronary artery; bar=50 μm (n=6). B, Quantification of the density of CCMR, NS; P>0.05, ***P<0.001 vs the PEDF group. C, Quantification of the mean diameter of CCMR, NS; P>0.05, **P<0.01, ***P<0.001 vs the PEDF group. D, Total cross-sectional area of CCMR, NS; P>0.05, ***P<0.001

vs the PEDF group. E, Confocal immunofluorescence analysis of the expression and distribution of VE-cadherin in CCMR vessels (bar=10 μm).

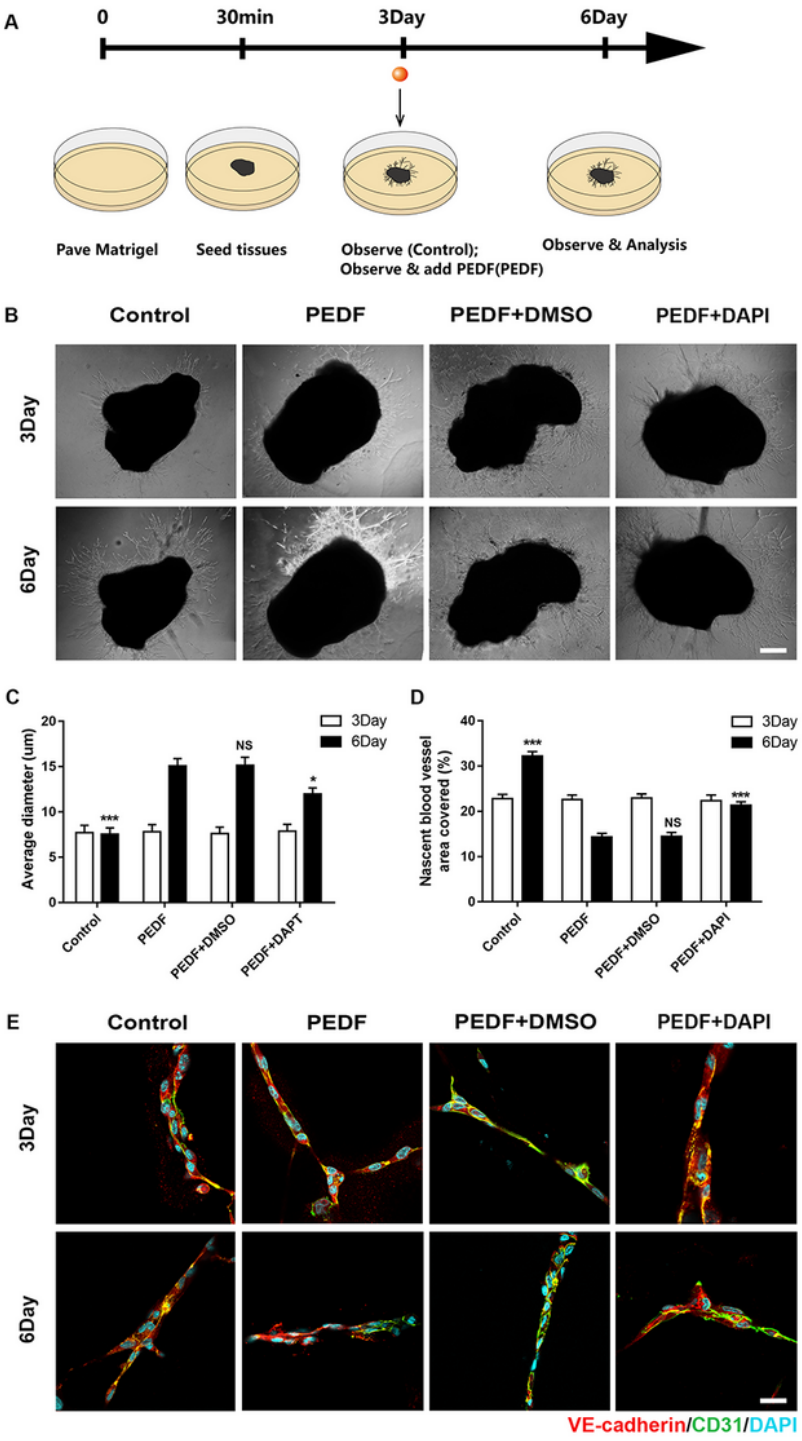

Figure 2

PEDF remodeling the nascent blood vessels is blunt in cardiac explants exposed to DAPT. A, Schematic diagram of the process of Cardiac explant Matrigel assay. B, Bright field images of cardiac explants (n=6), (bar=50 μm). C, Quantification of the average diameter of nascent vessels, NS; P>0.05, *P<0.05,

***P<0.001 vs the PEDF group. D, Quantification of density of area covered with nascent vessels, NS; P>0.05, ***P<0.001vs the PEDF group. E, Confocal immunofluorescence analysis of the expression and distribution of VE-cadherin in nascent vessels (bar=10 μm).

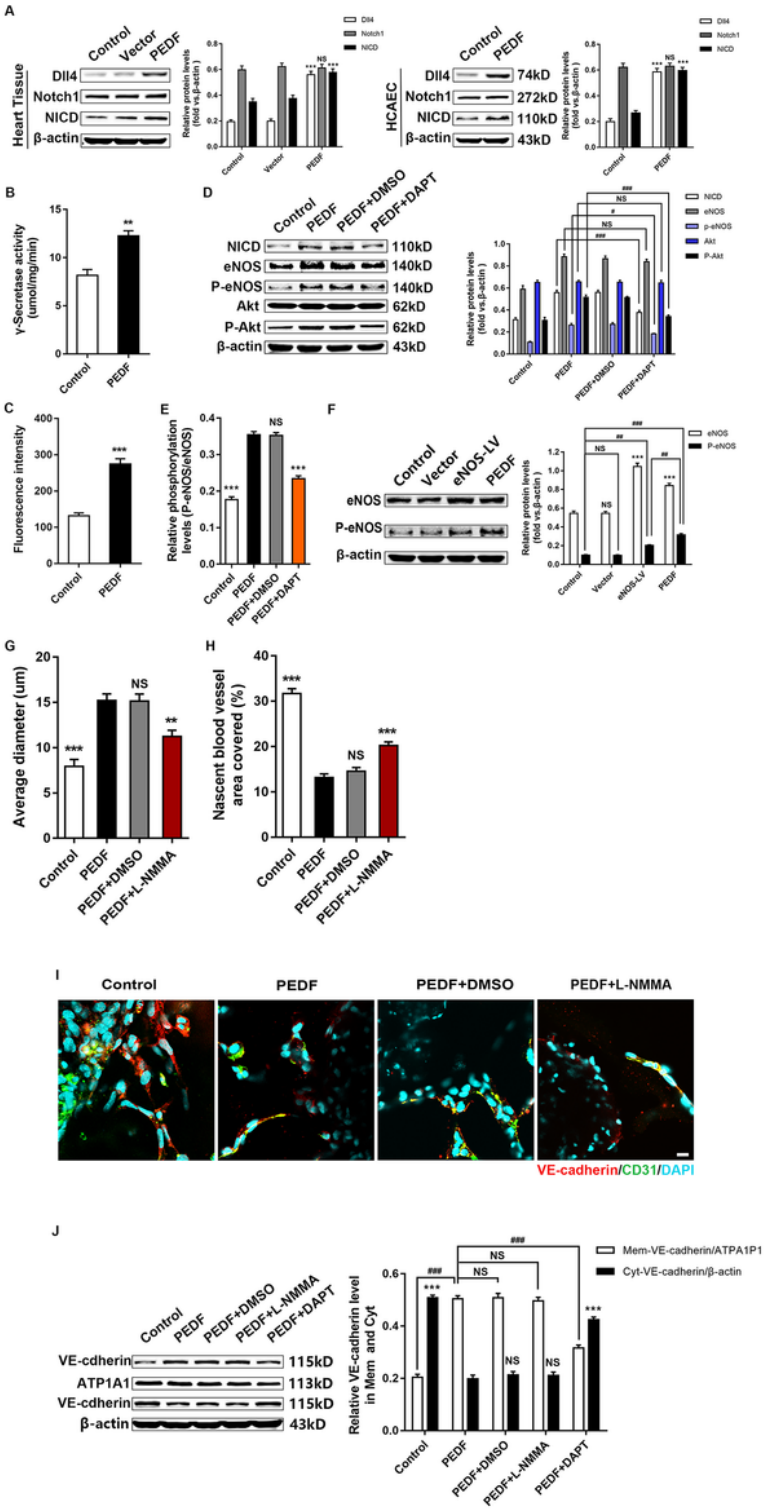

Figure 3

PEDF induces lumenal remodeling of nascent blood vessels via Notch1 / NO signaling pathway. A, Western blotting detected the levels of proteins related to Notch1 pathway in myocardial tissue and

HCAECs, NS; P>0.05, ***P<0.001 vs the control group, n=6. B, Detection of γ-secretase activity, **P<0.01 vs the control group, n=3. C, Detection of NO production, ***P<0.001 vs the control group, n=3. D and E, Determination of proteins related to the NO signaling pathway using western blot, NS; P>0.05, ***P<0.001 vs the PEDF group, #P<0.05, ###P<0.001 vs the indicated group, n=6. F, Expression of eNOS and P-eNOS proteins, NS; P>0.05, ***P<0.001 vs the Control group, ##P<0.01, ###P<0.001 vs the indicated group, n=6. G, Quantification of the average diameter of nascent vessels, NS; P>0.05, **P<0.01, ***P<0.001 vs the PEDF group, n=6. H, Quantification of density of area covered with nascent vessels, NS; P>0.05, ***P<0.001vs the PEDF group, n=6. I, Immunofluorescence analysis of the expression and distribution of VE-cadherin in nascent vessels (bar=10 μm). J, Quantitation of VE-cadherin in membrane and cytoplasm exposed to L-NMMA or DAPT, NS; P>0.05, ***P<0.001 vs the PEDF group, ###P<0.001 vs the indicated group, n=6.

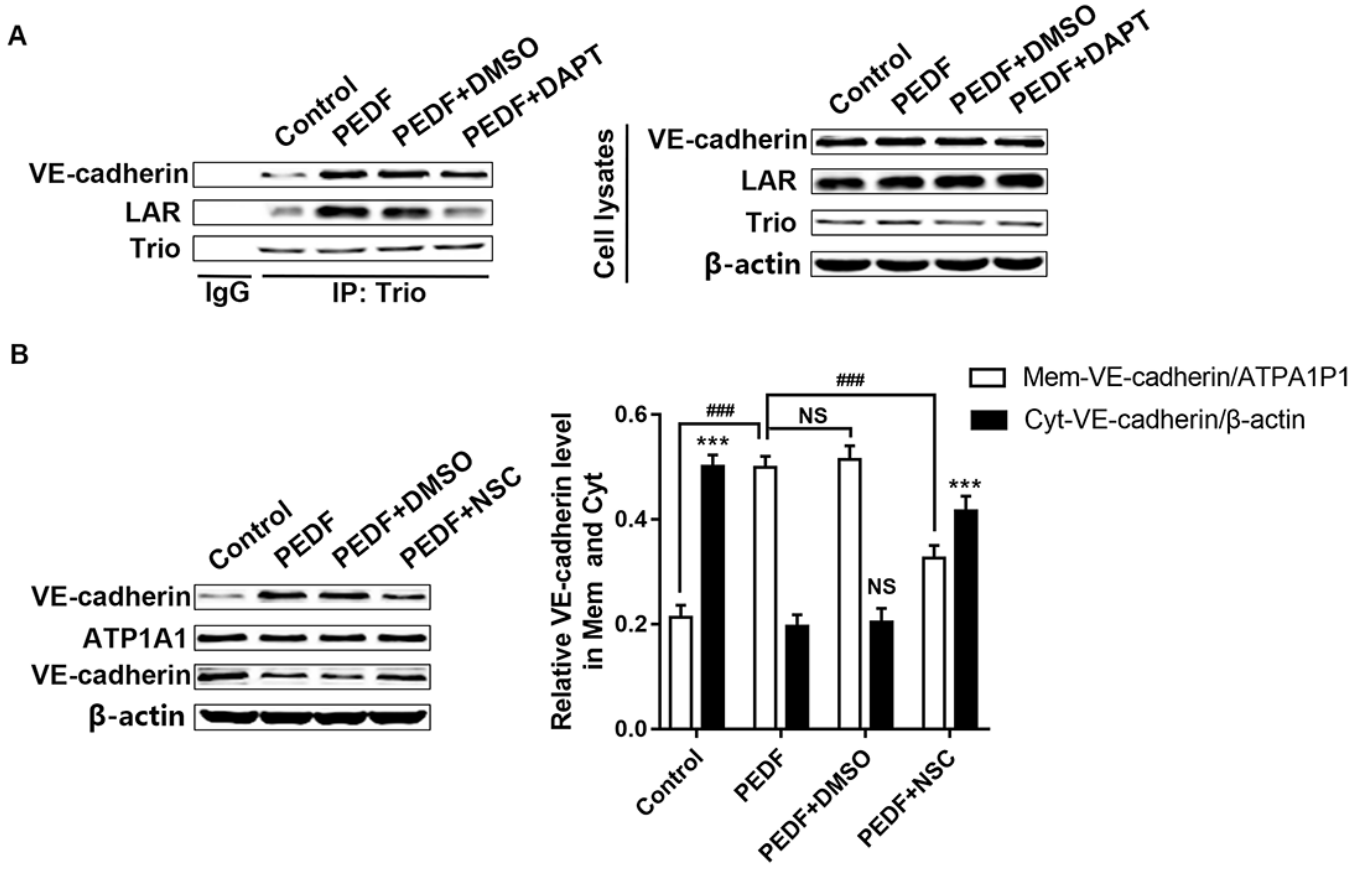

Figure 4

PEDF promotes assembly of adherens junctions in nascent blood vessels through non-canonical Notch1 signaling pathway. A, Detecting the formation of VE-cadherin/LAR/Trio complex by co-immunoprecipitation (n=3). B, Quantitation of VE-cadherin in membrane and cytoplasm exposed to NSC, NS; P>0.05, ***P<0.001 vs the PEDF group, ###P<0.001 vs the indicated group, n=6.

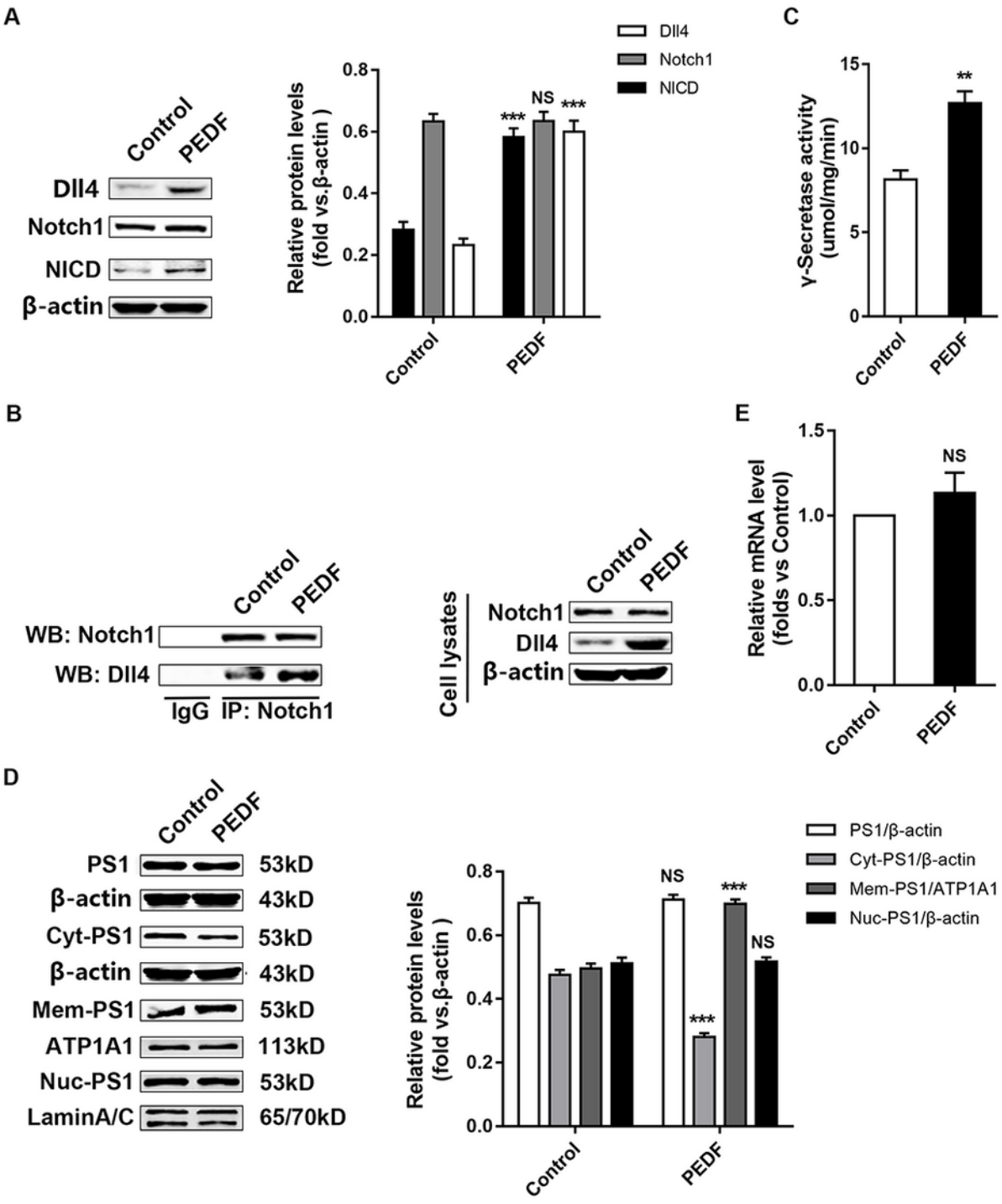

Figure 5

PEDF increases the expression of Dll4 protein and γ-secretase activity in endothelial cells. A, Proteins related to the Notch1 pathway were measured by western blot, NS; P>0.05, ***P<0.001 vs the control group, n=6. B, Detecting the formation of Dll4/Notch1 complex by co-immunoprecipitation (n=3). C, Detection of γ-secretase activity, **P<0.01 vs the control group, n=3. D, Western blot analysis of the

expression and distribution of PS1, NS; P>0.05, ***P<0.001 vs the control group, n=3. E, Relative gene expression of PS1 was quantified with qPCR, NS; P>0.05 vs the control group, n=3.

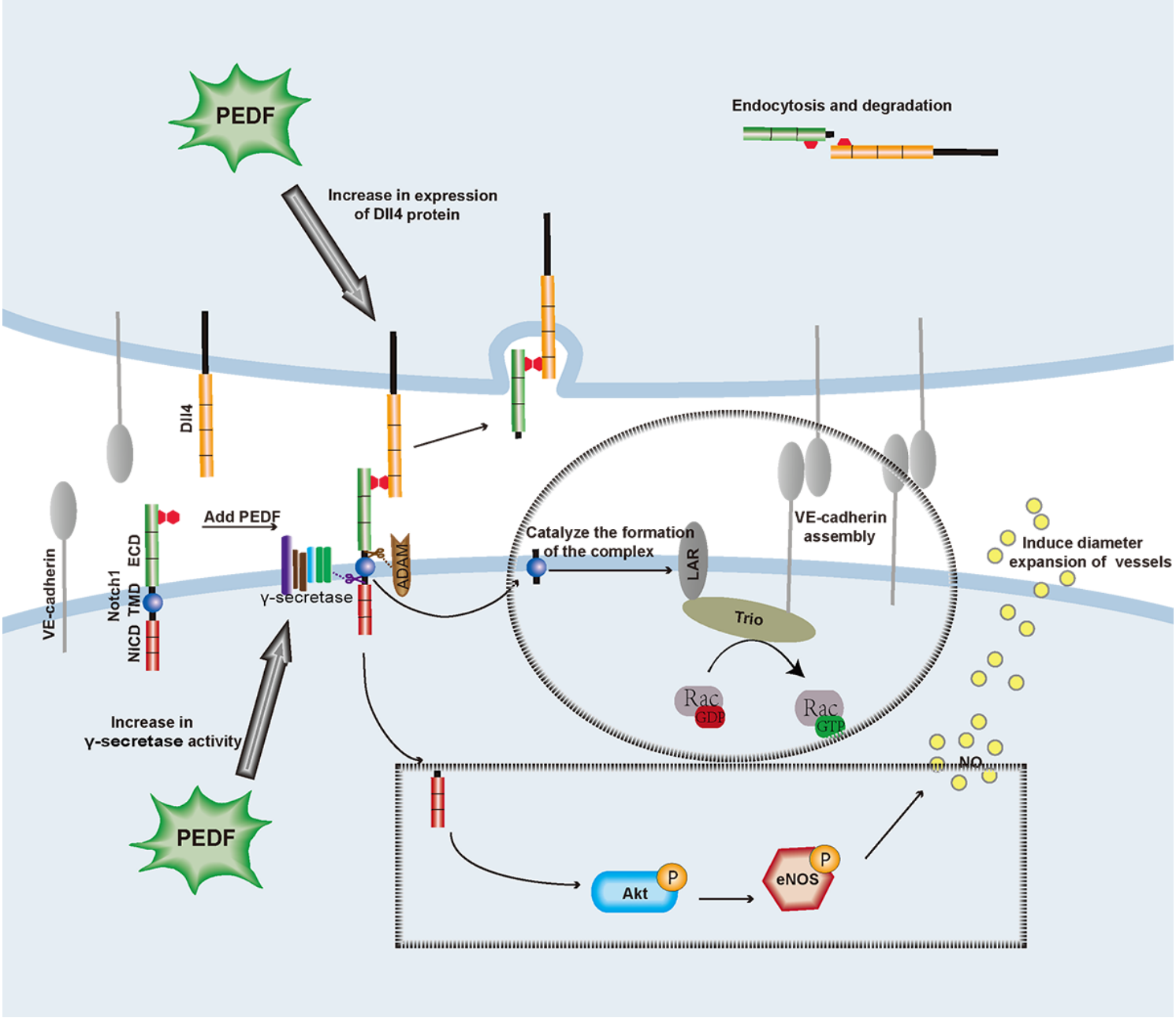

Figure 6

PEDF activates the Notch1 signaling pathway in endothelial cells. Dll4 protein expression and γ-secretase activity are increased in endothelial cells treated with PEDF. PEDF promotes the binding of Dll4 and Notch1, which is cleaved into ECD、TMD and NICD by the γ-secretase. TMD mediates the non-canonical Notch1 signaling pathway[16] responsible for VE-cadherin assembly (shown in the circle). PEDF promotes NO production through the NICD / Akt / eNOS pathway (shown in the rectangle).