Intelligent Pharmacy 4 (2026) 96–102

Contents lists available at ScienceDirect

Intelligent Pharmacy

journal homepage: www.keaipublishing.com/en/journals/intelligent-pharmacy

Full length article

Trophoblast-derived CD81 decreased eNOS expression in endothelial cell during preeclampsia development

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ARTICLE INFO

Keywords:
CD81
eNOS
Endothelial cell permeability
PI3K/Akt
Preeclampsia

ABSTRACT

Trophoblast shallow invasion and maternal vascular endothelial cell damage are recognized as key pathophysiological changes associated with preeclampsia (PE). Our previous study demonstrated that CD81 inhibits trophoblast cell invasion and induces endothelial cell dysfunction. In present study, we investigated whether trophoblast-derived CD81 influences endothelial cell permeability and the expression of endothelial nitric oxide synthase (eNOS) in endothelial cell, also we explored the related molecular mechanisms. Firstly, we observed that eNOS protein level was decreased in the placenta of patients with severe preeclampsia (sPE) by western blotting (WB) analysis. Immunohistochemical results indicated that eNOS expression was down-regulated in the villous of sPE patients. Secondly, we found that primary human umbilical vein endothelial cells (HUVECs) treated with Ad-CD8-media derived from HTR-8/SV neo cells exhibited increased cell permeability compared to those treated with Ad-CTL-media. Conversely, the cell permeability of siCD81-media treated primary HUVECs was inhibited. The expressions of eNOS, Akt S473 and PDK1 in HUVECs exposed to Ad-CD81-media from trophoblasts were reduced; while treated with siCD81-media, the expressions of these moleculars were elevated in HUVECs. Furthermore, using the inhibitor of PI3K/Akt, we discovered that LY294002 could significantly reverse the effects of CD81-related media on the expressions of eNOS, Akt S473 and PDK1 in the HUVECs. Finally, we found the expressions of Akt S473 and PDK1 were reduced in placenta of sPE patients by WB analysis. In conclusion, the present study indicated that trophoblast-derived CD81 decreased eNOS expression in endothelial cell and increased the ability of endothelial cell permeability. Furthermore, we revealed that downregulation of eNOS expression by trophoblast-derived CD81 may occur partly through the PI3K/PDK1/Akt pathway.

1. Introduction

Preeclampsia (PE) is a pregnancy-related complication characterized by the symptoms of hypertension and proteinuria. Recent studies suggest that this condition develops in two stages: in stage I, abnormal remodeling of uterine spiral arteries causes impaired placentation in the early pregnancy; subsequently, in stage II, vascular endothelial cell dysfunction is resulted by the release of molecules and factors from the placenta and the clinical manifestations are appeared in the second or third trimester.[1,2]

CD81 is recognized as a maker of extracellular vesicles, which can interact with different cell types and modulate cellular functions.[3] Wang et al. found that CD81 was upregulated in the exosomes isolated from urine and kidney of the PE model rat, influencing blood pressure regulation through modulation of salt/water balance.[4] Our previous study indicated that CD81 was increased in both the serum and placenta in the patients with severe PE (sPE) and inhibited trophoblasts invasion and affected vascular endothelial cell function.[5,6] CD81 is considered to be involved in the "two stages" of PE, but mechanism by which it influences endothelial cell function remains unclear.

Endothelial nitric oxide synthase (eNOS) plays a crucial role in regulating nitric oxide (NO) expression within endothelial cells and regulates the endothelial cell diastolic function and cell permeability ability. One report indicated that eNOS is decreased in the placenta of PE patients and eNOS-knockouted mice displaied PE-liked symptoms during pregancy development.[7,8] Also, research conducted by Min et al. found that the abilities of endothelial cells permeability and angiogenesis were damaged in eNOS-deficient mice.[9] Therefore, eNOS represents an important molecular player associated with the pathogenesis of PE.

The PI3K/Akt signal pathway is reported to be involved in the

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Peer review under the responsibility of Editorial Board of Intelligent Pharmacy.

https://doi.org/10.1016/j.ipha.2025.12.001
Received 18 November 2025; Received in revised form 4 December 2025; Accepted 6 December 2025
Available online 9 December 2025
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mechanism underlying the development of PE, as it participants the regulation of trophoblasts dysfunction induced by various factors.[10,11] Lin et al. found that CD81 influences the proliferation of hematopoietic stem cells by inhibiting the activation of Akt S473.[12] In the present study, we initially identified that the trophoblast-derived CD81 inhibited the expression of eNOS in the vascular endothelial cell. Subsequently, the etiology of regulation of eNOS expression by CD81 may be partly through the PI3K/PDK1/Akt signal pathway.

2. Materials and methods

2.1. Human placenta samples

We collected the human placenta samples of patients with sPE and the women with normal pregancy obtained from the Drum Tower Hospital, Nanjing, China. A case–control group (sPE vs Normal, $n = 10$) was set in 1:1 ratio. The patients with PE included in this study belonged to severe PE according to the criteria of PE in William's Obstetrics of the 24th version. The pregnant PE patients with chronic hypertension, cardiovascular diseases, gestational diabetes mellitus or chronic nephritis were excluded. The method of placenta collection has been described previously.[5] We collected the tissue about 2 cm away from the umbilical cord from the maternal side of the placenta, penetrating through the entire placenta. This study was approved by the Scientific Research Ethics Committee of the Drum Tower Hospital (2018-034-01 and 2021-214-01), and an informed consent was obtained from all of the participants.

2.2. Cell lines

The HTR-8/SV neo cells, which are derived from human first trimester extravillous trophoblast cells, were generously provided by Dr. Graham, Queen's University, Canada. The cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. RPMI 1640 medium (HyClone, Thermo Scientific, South Logan, USA) was supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone, Thermo Scientific), along with penicillin at a concentration of 100 U/mL and streptomycin at a concentration of 100 μg/mL.

2.3. Primary human umbilical vein endothelial cells (HUVECs)

Primary HUVECs were isolated from the umbilical vein vascular wall obtained from patients with normal pregancy using collagenase IA (1 mg/ml, Worthington Biochemical corporation, lakewood, NJ, USA). The tissue was treated for 10–15 min in 37 °C in a 5% CO_2 incubator. Subsequently, the cells were seeded onto plates coated with 0.15% gelatin and cultured in M199 medium (Gibco BRL/Invitrogen) containing 20% FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C in a 5% CO_2 incubator.

2.4. Generation of adenovirus, siRNA and recombinant CD81

The recombinant plasmids pCMV-CD81-3Flag-SV40-EGFP were transfected into HEK293A cells to generate adenovirus prestocks and then the recombinant adenovirus Ad-CD81 was purified. An adenovirus expressing GV314-CMV-Flag-GFP was used as the control (Ad-CTL). The siRNA targeting human CD81 (5′-AUCUGGAGCUGGGAGACAA-3′) and the siRNA negative control were synthesized by Ribobio (Guangzhou, China). These oligonucleotides were transfected into HTR-8/SV neo cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

Recombinant CD81 was generated by Ad-CD81-infected HTR-8/SV neo cells or siCD81-transfected cells. Firstly, HTR-8/SV neo cells had been infected with Ad-CD81 [100 multiplicity of infection (MOI)] or siCD81 (100 nmol/L) for 24 h, and then culture medium that contained recombinant CD81 protein was collected. The HUVECs received treatment consisting of recombinant CD81 mixed with cell culture medium in a volume ratio of 1:1. The LY294002, an inhibitor if PI3K/Akt, was applied to the HUVECs at a concentration of 40 μM.[13]

2.5. Immunohistochemistry

The placenta tissues intended for immunoperoxidase staining were fixed in 10% formalin-phosphate-buffered saline (PBS). Paraffin sections (5 μm) were prepared, de-waxed and hydrated. Endogenous peroxides were quenched using H_2O_2. Following heat-induced antigen retrieval, the sections were incubated with anti-eNOS (1:500, SC-654, santa-cruz) antibody, and then the sections were incubated with horseradish peroxidase-conjugated secondary antibody. Signals were visualized by incubation with diaminobenzidine (DAB). The sections were viewed using a Leica DM2000 microscope.

2.6. WB analysis

WB analysis was performed using standard procedures. The placenta tissues and cultured cells that have been washed twice by PBS were lysed in RIPA lysis buffer containing protease and phosphatase inhibitor cocktails (MedChemExpress, USA). The protein concentrations were assessed with the BCA Protein Assay Kit (Thermo Scientific Pierce). The proteins were separated by SDS-PAGE gels and subsequently transferred to PVDF membranes. After blocking in 5% nonfat milk (Bio-Rad, USA) for 1 h, the membranes were incubated overnight at 4 °C with primary antibodies: eNOS (1:1000, SC-7637, santa-cruz), PDK1(1:1000, Bioworld), Akt(1:1000, Bioworld), Akt S473(1:1000, Bioworld),GAPDH (1:1000, Bioworld) or α-tubulin(1:5000, Bioworld). The membranes incubated with a peroxidase-conjugated secondary antibody for 1 h at room temperature. Signal visualization was performed using an ECL kit (Bio-Rad, USA). The bands were quantified using Image J. We implemented three independent replicate experiments.

2.7. Quantitative real-time PCR (qRT-PCR)

The qRT-PCR was employed to measure the mRNA level of eNOS in HUVECs treated with recombinant CD81. In brief, total RNA (2 μg) was extracted from cells with Trizol (Invitrogen, Carlsbad, CA, USA) and was reverse-transcribed into cDNA in a 20 μL reaction volume utilizing random primers on a MyiQ Single Color Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The primers were as follows: hsa-eNOS-F:CCCTTCAGTGGCTGGTACAT, hsa-eNOS-R:CACGATGGTGACTTTGGCTA; hsa-18sRNA-F: CGGCTACCACATCCAAGGAA, hsa-18sRNA-R:CTGGAATTACCGCGGCT. The data obtained from three independent experiments were used for analysis of relative gene expression using the $2^{-\Delta\Delta}$CT method. We implemented three independent replicate experiments.

2.8. Cell permeability assay

HUVECs were plated at a density of 3 × 10^5 cells/well onto transwell filters (0.4 μm pore size, Corning, USA) and cultured with 100 μL of M199 medium in the upper chamber and 500 μL of media in the lower chamber for 12 h. Then, the cells were cultured with the recombinant CD81 at a volume ratio of 1:1 for an additional 48 h. Following this incubation period, 100 μL FITC-conjugated dextran (1 mg/mL, Sigma) diluted with opti-MEM was added to the upper chambers. Specimens measuring 50 μL each were collected from the lower chamber at time points of 15, 30, 45, 90, and 120 min respectively. Of note, 50 μL of fresh medium was added to each well in order to maintain the liquid level on both sides. Fluorescence intensity was measured using a spectrophotometer set to an excitation wavelength of 485 nm and an emission wavelength of 535 nm. We implemented three independent replicate experiments.

Table 1
Clinical characteristics of patients with sPE and normal pregnant women.

Characteristics	Normal ($n = 10$)	sPE ($n = 10$)	*P*-value
Maternal age (years)	29.8 ± 2.3	28.7 ± 4.6	*n.s.*
Gestational age (weeks)	39.4 ± 1.5	37.2 ± 2.3	<0.05
Systolic BP (mmHg)	120.7 ± 3.9	159.1 ± 10.6	<0.05
Diastolic BP (mmHg)	70.0 ± 4.3	102.6 ± 4.7	<0.05
Proteinuria	–	+ – ++++	<0.05
Fetal weight (g)	3251.0 ± 361.5	2597.0 ± 659.9	<0.05
Placenta weight (g)	553.0 ± 29.1	540.8 ± 137.8	*n.s*

2.9. Statistical analysis

Data that followed a normal distribution were presented as mean ± standard deviation (SD). Differences between means were assessed using a two-tailed Student's *t*-test, conducted with SPSS version 22.0 software. *P*-values less than 0.05 were considered indicative of statistically significant differences.

3. Results

3.1. eNOS expression was down-regulated in the placenta of patients with sPE

We analyzed the human placenta samples, 10 from patients with sPE and 10 from gestational age-matched normal pregnant women, and clinical characteristics of these patients were listed in Table 1. There were no significant difference on the maternal age between the two groups. Immunohistochemical analysis showed that eNOS expression was significantly reduced in the villous of sPE patients compared to the normal patients, while no significant difference was observed in its expression on the basal plate (BP) between the two groups (Fig. 1A). Additionally, compared with the normal group, we found that the protein level of eNOS was lower in the placenta lysis of sPE patients using WB analysis (Fig. 1B and C). These findings indicated that eNOS expression was down-regulated in patients suffering from sPE.

3.2. Trophoblast-derived CD81 reduced eNOS expression and increased endothelial cell permeability in HUVECs

We investigated the impact of trophoblast-derived CD81 on the expression of eNOS in endothelial cells using the recombinant CD81. The CD81 expressions of the recombinant CD81 was confirmed (Supplementary Fig. 1). As showed in Fig. 2A and B, both the mRNA and protein expressions of eNOS in HUVECs treated with Ad-CD81-media from the HTR-8/SV neo cells were decreased compared to those treated with Ad-CTL-media. Meanwhile, using the siCD81-media, we discovered that the levels of CD81 mRNA and protein were up-regulated in siCD81-media-treated HUVECs (Fig. 2C and D). Thus, the above results prompted trophoblast-derived CD81 reduced eNOS expression in endothelial cells.

The activation of vascular endothelial cells is associated with the increased ability of the cell permeability during PE development. We then researched the permeability of endothelial cells dealing with the

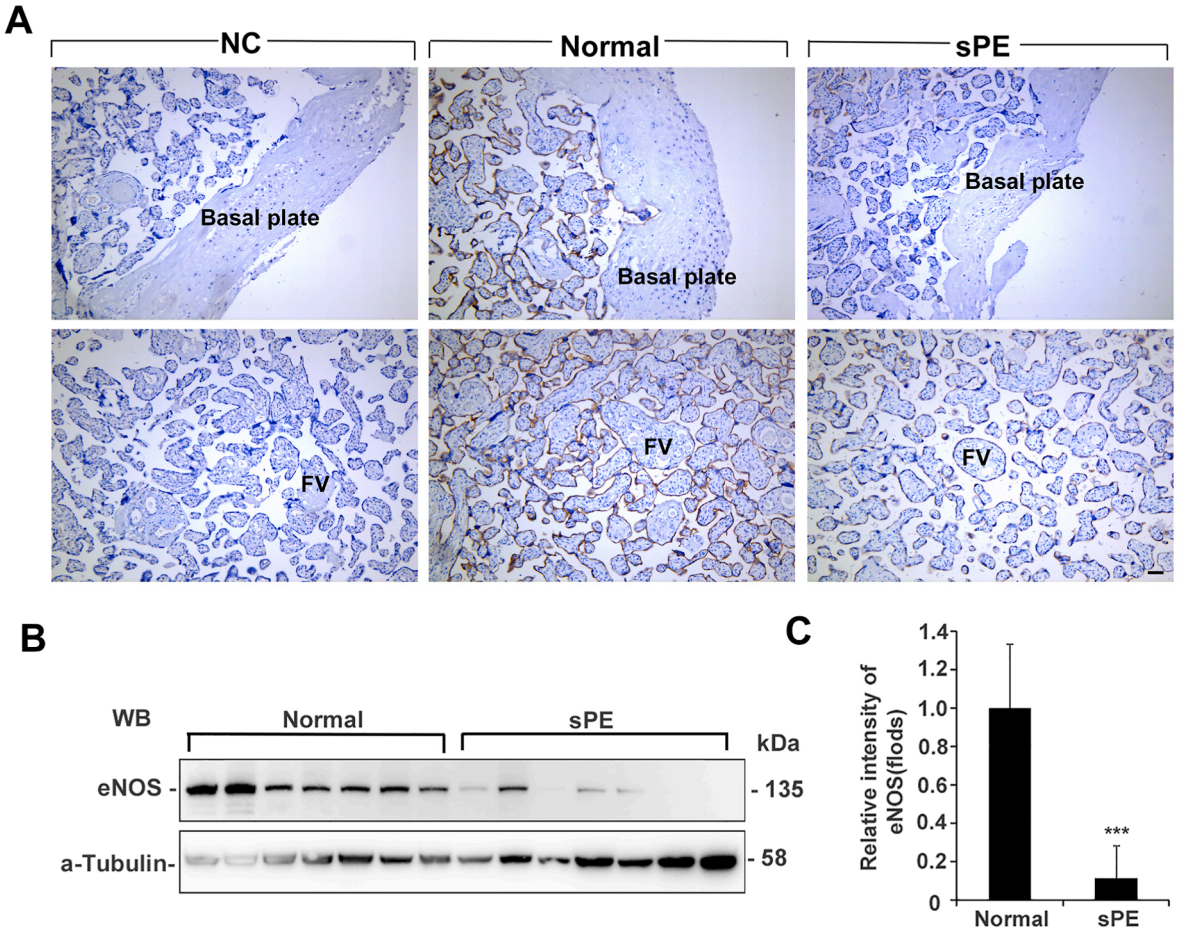


Fig. 1. eNOS was down-regulated in the placenta of patients with sPE (A) Placental tissue sections were stained with anti-eNOS antibody by immunohistochemistry. BP: basal plate; FV: floating villi; NC: negative control. Bar: 25 μm (B, C) Immunoblotting of the placenta tissue showed that eNOS protein level was reduced in samples from patients with sPE. sPE vs. Normal: $p < 0.01$, $n = 10$.

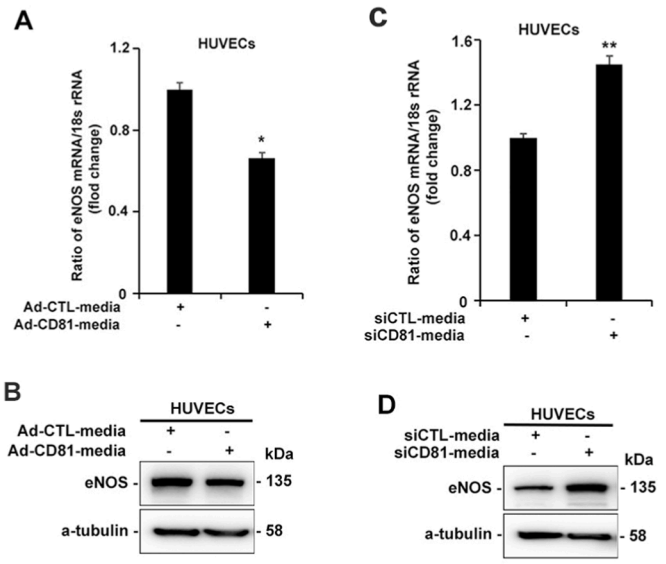


Fig. 2. Trophoblast-derived CD81 reduced eNOS expression in HUVECs (A, B) The levels of eNOS mRNA and protein were decreased in the HUVECs treated with Ad-CD81-media using the qRT-PCR and WB analysis. Ad-CD81 vs. Ad-CTL: $p < 0.05$ (C, D) The levels of eNOS mRNA and protein were elevated in HUVECs treated with siCD81-media. siCD81 vs. siCTL: $p < 0.01$.

recombinant CD81. Compared to Ad-CTL-media-treated cells, the fluorescence intensity was markedly increased following the time in HUVECs treated with Ad-CD81-media (Fig. 3A). Conversely, as illustrated in Fig. 3B, the fluorescence value was reduced in the siCD81-media-treated HUVECs. The above results suggested that trophoblast-derived CD81 enhanced endothelial cell permeability.

3.3. Trophoblast-derived CD81 negatively mediates the PI3K/Akt signaling pathway by downregulating PDK1 in HUVECs

Several studies have reported CD81 is involved in the mediation of PI3K/Akt signaling pathway.[9,14] Therefore, we tested whether the upregulated CD81 in trophoblasts could inhibit the PI3K/Akt signaling pathway in endothelial cells. Using WB analysis, phosphorylation at Akt Ser473 was decreased in HUVECs dealed with Ad-CD81-media cells (Fig. 4A). On the other hand, as displaied in Fig. 4B, the expression of Akt Ser473 was upregulated in siCD81-media-treated HUVECs. Then the endothelial cells were pre-treated with LY294002, an inhibitor of PI3K/Akt pathway. Fig. 4C and D demonstrated that the expressions of both eNOS and Akt Ser473 were reversely decreased in the siCD81-media-treated HUVECs.

Next, we sought to elucidate the molecular mechanism through which CD81 inhibited PI3K signaling pathway. Since PIP3-activated PDK1 directly phosphorylates two conserved residues on Akt Ser473 and Thr308, we next asked whether this downstream effector of PI3K is involved in CD81 signaling. Notably lower protein level of PDK1 was observed in Ad-CD81-media-treated HUVECs compared to those infected with Ad-CTL-media (Fig. 5A). The opposited phenomenon was observed at PDK1 protein level in siCD81-media-infected HUVECs (Fig. 5B). Furthmore, as indicated in Fig. 5C, LY294002 reversed the increasing PDK1 expression in the siCD81-media-treated endothelial cells. Then total proteins were extracted from placenta tissues from patients with sPE and normal pregnant women. Compared with control placentas, PDK1 expression was lower in the placenta from patients with sPE (Fig. 5D,F). In addition, phosphorylation of Akt Ser473 was reduced in the placenta of patients with sPE (Fig. 5E,G), corroborating the upregulation of CD81 observed within PE placenta samples.

Taken together, these in vitro and *in vivo* data indicated that trophoblast CD81 inhibited the PI3K/Akt signaling pathway by downregulating the expression of PDK1.

4. Discussion

The theory of "two stage of PE development" posits that the impaired placentation in the early pregancy released toxic factors, leading to the onset of clinical manifestations of maternal and fetal during the third trimester. Our previous study found that CD81 was increased in both the serum and placenta in the patients with sPE, indicating that CD81 may function through autocrine and exocrine.[5] In the present report, we demonstrated that trophoblast-derived CD81 reduced eNOS expression in HUVECs and increased endothelial cell permeability. Furthermore, we reveled that trophoblast-derived CD81 downregulated the expressions of Akt S473 and PDK1 in the endothelial cells. Finally, the expressions of Akt S473 and PDK1 were decreased in the placenta of patients with sPE.

The dysfunction of vascular endothelial cells is considered to be a pathological change of PE. eNOS in an important molecular involved in the regulating of endothelial cell functions, including cell contraction, diastole and cell permeability.[15,16] Previous studies have accumulated evidence that decreased eNOS expression has been detected in the placentas and sera of patients with PE.[7,17] Our present study in agreemently verified eNOS was reduced in PE patient placenta. Immunohistochemistrial result indicated that eNOS expression was significantly decreased in the floating villi of PE patients. The floating villi can directly contacted with the maternal serum, allowing for the delivery of various molecules and factors via extracellular microvesicles. We previously determined that CD81 was upregulated in the serum and serum exosome of the patients with sPE, indicating that the role of secreted CD81, although the limitation of existing clinical detection methods of CD81. In the present report, we revealed that trophoblast-derived CD81 reduced the eNOS mRNA and protein levels in HUVECs and promoted the endothelial cell permeability. We firstly pointed out the trophoblast CD81 regulated the eNOS expression of the endothelial cell. The other

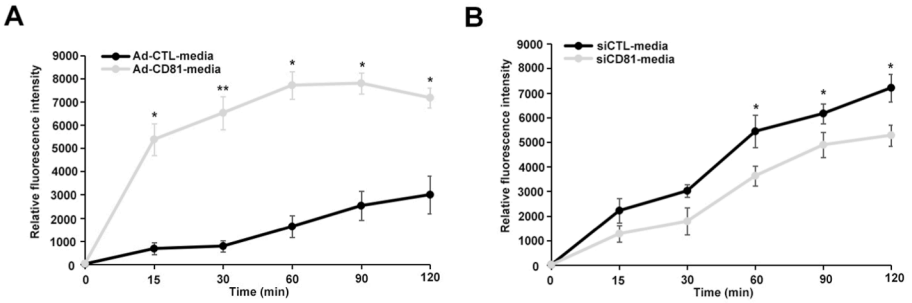


Fig. 3. Trophoblast-derived CD81 increased endothelial cell permeability in HUVECs (A) The cell permeability was increased in the HUVECs treated with Ad-CD81-media by the cell permeability assay. *Ad-CD81 vs. Ad-CTL: $p < 0.05$; **Ad-CD81 vs. Ad-CTL: $p < 0.01$ (B) The cell permeability was reduced in the HUVECs treated with siCD81-media. *siCD81 vs. siCTL: $p < 0.01$. *$p < 0.05$; **$p < 0.01$.

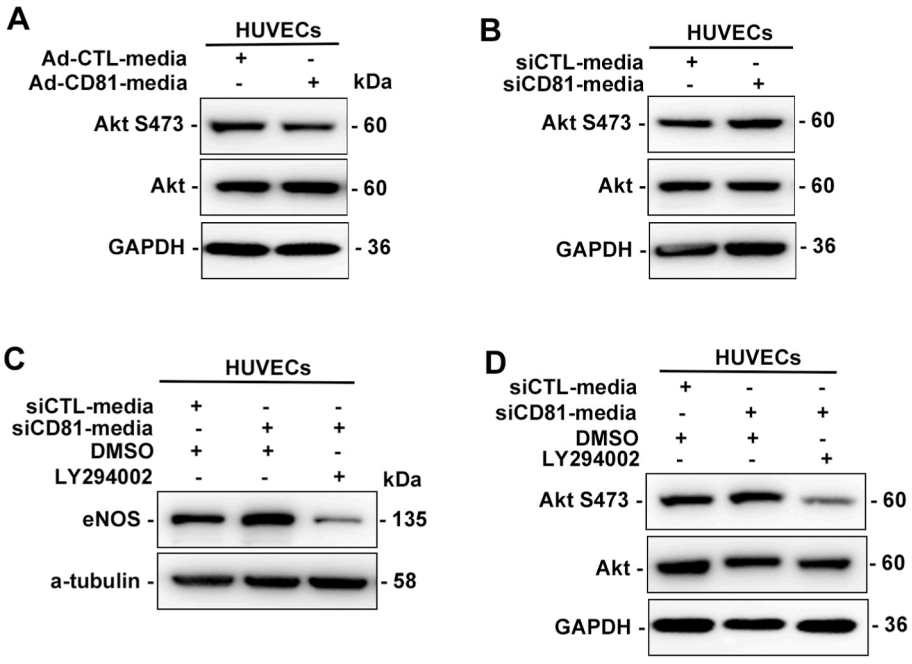


Fig. 4. Trophoblast-derived CD81 down-regulated PI3K/Akt signaling pathway in HUVECs (A, B) The expression of p-Akt S473 was detected in the HUVECs treated with Ad-CD81-media or siCD81-media by WB analysis (C, D) Pre-processing the HUVECs with LY294002, then the expressions of eNOS and p-Akt S473 were detected in the HUVECs treated with Ad-CD81-media or siCD81-media by WB analysis. The concentration of LY294002 was 40 μM.

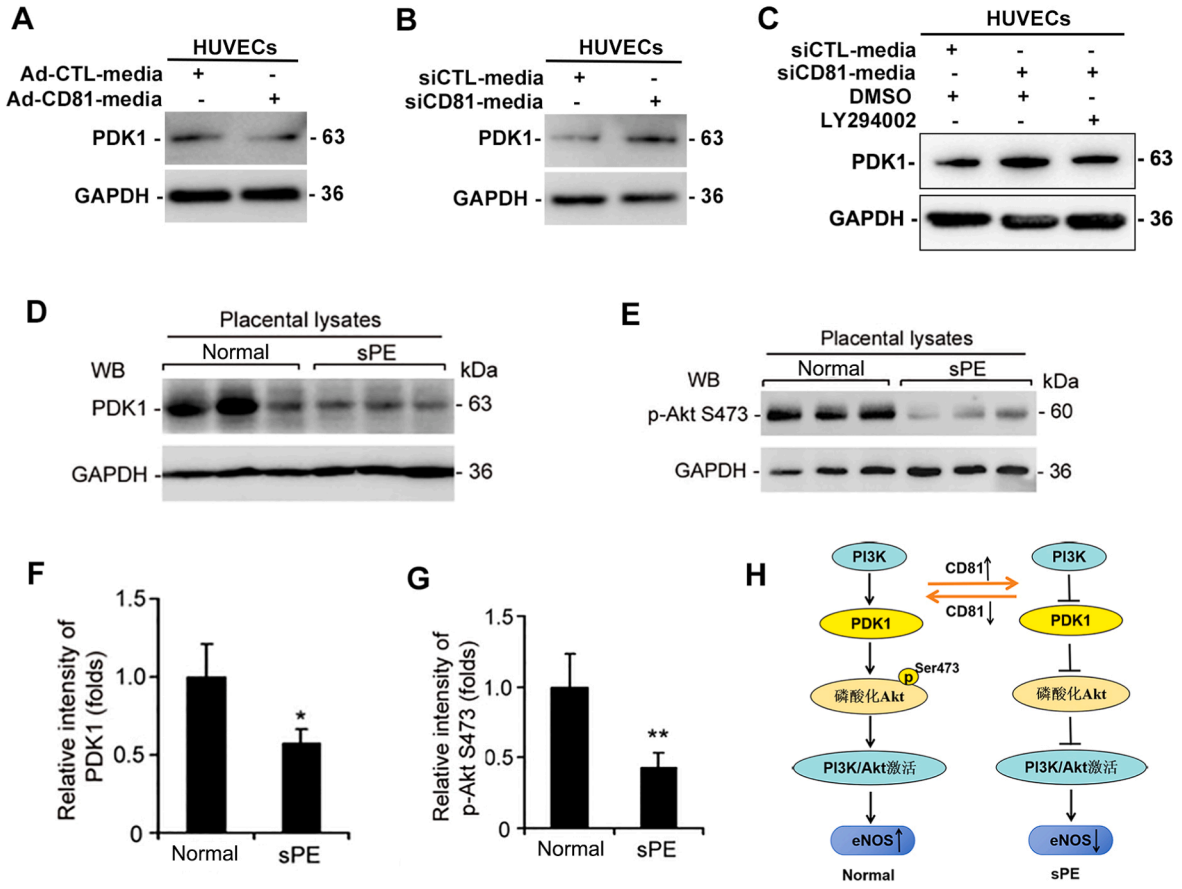


Fig. 5. Trophoblast-derived CD81 negatively mediates the PI3K/Akt signaling pathway by downregulating PDK1 in HUVECs (A, B) The expression of PDK1 was detected in the HUVECs treated with Ad-CD81-media or siCD81-media by WB analysis (C) Pre-processing the HUVECs with LY294002, the expression of PDK1 was detected in the HUVECs treated with Ad-CD81-media or siCD81-media by WB analysis. The concentration of LY294002 was 40 μM (D, F) PDK1 expression was downregulated in the placental lysates from patients with sPE (sPE vs Normal: $p < 0.05$, $n = 10$). The relative intensity of PDK1 was assessed by Image J (E, G) Akt Ser473 phosphorylation was downregulated in the placenta lysates from patients with sPE (sPE vs Normal: $p < 0.01$, $n = 10$). The relative intensity of Akt Ser473 was assessed by Image J (H) The molecular mechanism of CD81 involvement in the pathogenesis of sPE.

dysfunctions such as endothelial cell information and cell diastole may need to explore by furthermore research.

To understand the molecular mechanisms underlying the CD81-mediated eNOS expression, we assessed the effect of trophoblast CD81 on the PI3K signaling pathway in endothelial cell. Several reports expressed that PI3K signaling pathway is involved in regulation of trophoblast cell function and is implicated the pathogenesis of PE.[18–20] Our data demonstrated that trophoblast-derived CD81 repressed this pathway in HUVECs by reducing Akt phosphorylation at Serine 473 residue. In consistent with our result, PI3K/Akt/eNOS signaling pathway is reported to be related to the coronary microvascular dysfunction.[21] Xu et al. found that ANXA4 raised the ability of trophoblast invasion through the adjusting of PI3K/Akt/eNOS pathway.[22] Intrigued by CD81 regulated PI3K/Akt signaling pathway, we addressed the molecular basis of this regulation and determined how CD81 interferes with PDK1, a downstream effector of PI3K. Strikingly, we found HUVECs infected with Ad-CD81-meida downregulated PDK1 protein expression and the siCD81-meida had the opposite effect on PDK1 level. The results of placenta tissue of PE patients also confirmed the downregulation of PDK1 and Akt S473 *in vivo*. Mainly researches indicated that PI3K/Akt is inactivated during PE development.[23–25] Liu et al's report indicated that inhibition of the PI3K/Akt Pathway as a promising therapeutic approach for PE.[26] To the best of our knowledge, this is the first demonstration of the molecular mechanism by which CD81 inactivates Akt. Therefore, these data describe a novel molecular mechanism for the regulation of PI3K by CD81 and provide an insight into the mechanism by which CD81 exerts inhibitory effects on target cells. By downregulating PDK1 to inhibit Akt activation, a multitude of downstream signals could be interrupted. Although the molecular details underlying this observation remain to be elucidated, simplistic explanatory models can be envisioned (Fig. 5H). One limitation of our study was lacking the animal experiments to verify the regulation of CD81 on eNOS expression in endothelial cell. The molecular expressions were detected in the placenta tissue from patients with late-onset severe PE in the present study. Thus, the functional differences of the CD81/eNOS pathway in different subtypes of PE cannot be clarified. In recent years, peptides have been recognized as a treasure trove for drug discovery[27–30], and the identification of peptides targeting CD81 and their possible applications might be a promising field in the future research.

In conclusion, our present study firstly reports that trophoblast CD81 reduced eNOS expression and increased cell permeability in endothelial cell. In vitro results confirm that CD81 inhibits eNOS expression through repressing PDK1-mediated PI3K/Akt signaling pathway. Our findings indicated that CD81 is involved in the two stages of PE development and it may be a potential biomarker for early diagnosis of PE and the possible candidates targeting CD81 may be explored to deal with the diseased in the future.

CRediT authorship contribution statement

Li Shen: Writing – original draft, Methodology, Formal analysis, Data curation. **Yujing Li:** Validation, Methodology, Data curation. **Dan Liu:** Methodology, Data curation. **Qiang Li:** Writing – review & editing, Validation, Conceptualization.

Data availability

All data supporting the findings of this study are available with the paper and its Supplementary Information.

Declaration of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

We thank Dr Charles Graham from Queen's University, Kingston, Ontario, Canada for gifting HTR-8/SV neo cell.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ipha.2025.12.001.

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