Potential mechanism of transient receptor potential cation channel subfamily V member 1 combined with an ATP-sensitive potassium channel in severe preeclampsia

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Abstract. Severe preeclampsia is one of the most serious obstetric diseases. However, the pathogenesis of the disease is not fully understood. In the present study, placental artery and blood serum was collected from patients with severe preeclampsia, as well as from normal pregnant women. The results of reverse transcription-quantitative (q)PCR, western blotting, and immunohistochemical staining revealed markedly decreased transient receptor potential cation channel subfamily V member 1 (TRPV1), ATP-sensitive potassium channel (KATP) subtype Kir6.1/SUR2B and endothelial nitric oxide synthase (eNOS) expression in severe preeclampsia tissue specimens compared with those in samples from normal pregnant women. The nitrate reduction method indicated lower NO levels in the tissue specimens and serum of patients with severe preeclampsia. Moreover, hematoxylin-eosin staining showed that the endothelial cell layer in the placental artery of patients with severe preeclampsia was notably damaged. To investigate the potential role of TRPV1-KATP channels in severe preeclampsia, HUVECs were used for *in vitro* experiments. The samples were divided into a control group, a TRPV1 agonist group (capsaicin) and a TRPV1 inhibitor group (capsazepine). qPCR and western blotting revealed that the relative gene and protein expression levels of TRPV1, Kir6.1, SUR2B and eNOS in the control group were significantly lower than those in the capsaicin group and considerably higher than those in the capsazepine group. Based on previous studies and the results of the present study, we hypothesized that impairment of the endothelial TRPV1-KATP channels results in decreased eNOS/NO pathway activity, which may be one of the mechanisms involved in severe preeclampsia. The increase in NO generation mediated by TRPV1-KATP may be a suitable target for the management of severe preeclampsia.

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Introduction

Severe preeclampsia is one of the most serious hypertensive disorders of pregnancy; it is characterized by a persistent increase in blood pressure after 20 weeks of gestation, often alongside maternal organ function impairment and fetal-placental complications (1). The pathogenesis of this disease has not been fully elucidated. The fundamental pathophysiological changes are systemic vasospasm and endothelial injury, but the pathogenesis of severe preeclampsia caused by vascular endothelial dysfunction is still unclear (2). Transient receptor potential cation channel subfamily V member 1 (TRPV1) has attracted increasing attention in the study of the cardiovascular system in recent years (3-5). A study showed that dietary capsaicin could activate TRPV1 on endothelial cells and promote the endothelial nitric oxide synthase (eNOS)/NO pathway, which may enhance endothelial production of NO and reduce blood pressure in rats with spontaneous hypertension (6). The ATP-sensitive potassium channel (KATP), a specific type of voltage-dependent potassium ion channel, is composed of an inwardly rectifying potassium channel (Kir) and sulfonylurea receptor (SUR), and the SUR2B/Kir6.1 subtype is also known as the vascular type (7). Studies have shown that the KATP channel-specific agonist etakarin not only directly relaxes vascular smooth muscle, but also acts on endothelial cells to increase eNOS expression and promote the synthesis and release of NO (8,9). Current correlations have been reported between transient receptor potential channels and potassium ion channels (4,10,11). A study confirmed the involvement of large-conductance K+ (BKca) in coronary endothelium-dependent relaxation mediated by TRPV1 (4); however, to the best of our knowledge, there are no reports on the role of TRPV1 and the KATP subtype SUR2B/Kir6.1 in severe preeclampsia. Since the placenta is a vital tissue that connects the fetus to the mother, and once the placenta has been delivered, this illness may be rapidly treated; there is no

doubt that the placenta has an essential role in the development of this disease (12). As one of the vasoactive substances regulating blood flow homeostasis in the body, NO must also be one of the crucial factors regulating blood flow perfusion in the placental artery (13). Therefore, the present study aimed to investigate whether severe preeclampsia was associated with impaired vasodilation mediated by TRPV1-KATP channels in the placental artery, leading to deficient eNOS/NO pathway activity and severe maternal vascular endothelial dysfunction.

Materials and methods

Management of human placental arteries. The present study was approved by the Clinical Trial Ethics Committee of the Affiliated Hospital of Southwest Medical University (approval no. KY2019039). All work was performed in accordance with the provisions of the Declaration of Helsinki and its later amendments. All patients provided written informed consent for specimen collection. A total of 10 pregnant women with a singleton pregnancy diagnosed with severe preeclampsia were selected as the experimental group (SP group), whose average maternal age ranged from 23 to 41 years, with a mean age of 32.20±5.226 years. In addition, 10 pregnant women with a singleton pregnancy with normotensive pregnancies were selected as the control group (NP group), whose average maternal age ranged from 22 to 44 years, with a mean age of 30.40±5.475 years (Table I). All patients were hospitalized and delivered by either vaginal delivery or cesarean section at the Department of Obstetrics, The Affiliated Hospital of Southwest Medical University (no. 8, Section 2, Kangcheng Road, Luzhou, Sichuan, China) between May 2020 and May 2021. The inclusion criteria for the SP group were based on the American College of Obstetricians and Gynecologists Guidelines (14). Subjects were excluded if they had chronic hypertension, kidney disease, cardio-cerebrovascular disease, severe liver and kidney function impairments, other primary diseases, systemic diseases or other pregnancy complications. Patients with a history of smoking, alcohol abuse, syphilis, hepatitis virus or human immunodeficiency virus were also excluded. Blood samples were acquired from the mother by peripheric venous puncture before any medical treatments were administered. Samples were then placed in anticoagulant tubes, centrifuged at 3,000 x g for 15 min at room temperature and stored in a -20˚C refrigerator until they were used for extraction. After the placenta was delivered, the fetal membrane was immediately stripped under sterile conditions, and 3-5 pieces of placental tissues with a small placental artery near the edge of the placenta, ~4x1x1 cm^3 in size, were extracted and placed in the prepared specimen box, which was immediately transferred to the laboratory under low-temperature conditions. Next, the perivascular connective tissue was rapidly and gently removed under a microscope, taking care to minimize the damage to the blood vessels, and vessels with diameters of 0.1-0.2 cm and lengths of 2-3 cm were separated. Some of the isolated blood vessels were frozen in liquid nitrogen and then quickly transferred to a -80˚C freezer for cryopreservation, and these samples were used for subsequent PCR and western blot analyses, and the nitrate reductase method. The remainder were fixed in 4% paraformaldehyde for >24 h at room temperature, and the specimens were used for subsequent hematoxylin-eosin staining and immunohistochemical analysis.

Hematoxylin-eosin staining of the placental arteries. Hematoxylin and eosin (H&E) staining was performed as previously described (15,16). To deparaffinize the paraffin sections, slides were submerged for 5 min in a slide jar filled with xylene (Sinopharm Chemical Reagent Co., Ltd.), repeating this procedure twice and using fresh xylene each time. Subsequently, sections were rehydrated by running slides through a series of decreasing concentrations of EtOH (Sinopharm Chemical Reagent Co., Ltd.). Hematoxylin (Shanghai Jingke Chemical Technology Co., Ltd.) was used to stain the tissues for 5 min at room temperature, and after washing with tap water, differentiation in staining was visualized by submerging for 30 sec in 1% acetic acid. To visualize the blue staining, the sections were rinsed under running tap water for 5 min, after which the slices were dyed in eosin solution (Shanghai Jingke Chemical Technology Co., Ltd.) for 2 min at room temperature. After dehydration with EtOH and transparency with xylene, the slices were sealed with neutral balsam (G8590-100; Solarbio). Finally, tissues were imaged under an electron microscope (400x magnification; OLYMPUS CX-21; Shanghai Xinyu Biotechnology Co., Ltd.).

Cell culture. HUVECs (cat. no. CL-0675; Procell Life Science & Technology Co., Ltd.) were cultured in high-glucose DMEM (AMEKO; cat. no. FB8025; Shanghai Lianshuobaowei Biological Technology Co., Ltd.) supplemented with 10% FBS (Guangzhou Ruite Biological Technology Co., Ltd.) and 1% penicillin and streptomycin (Shanghai Biyuntian Biotechnology Co., Ltd.) at 5% CO_2 and 37˚C in a humidified chamber. HUVECs in the logarithmic growth stage were subcultured and inoculated in six-well plates. The cells were divided into a control group, a capsaicin group (cat. no. HY-10448; MedChemExpress) and a capsazepine group (cat. no. HY-15640MCE; MedChemExpress). After reaching ~80% confluency, high-glucose DMEM containing capsaicin and capsazepine, prepared in DMSO to a final concentration of 1 μmol/l, was added. This concentration was based on a previous study by Yang *et al* (6), in which NO production was highest with 1 μM capsaicin in cultured endothelial cells from Wistar rats. In the control group, high-sugar DMEM with an equivalent volume of DMSO was used. After 24 h of incubation, total RNA and protein were extracted from the cells.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from tissues and cells using TRIzol reagent (cat. no. 15596018; Thermo Fisher Scientific, Inc.), the absorbance of the extracted RNA was measured using a spectrophotometer on a Bio-Rad 3000 UV-vis spectrophotometer (Bio-Rad Laboratories, Inc.), and the integrity was examined by gel electrophoresis (Bio-Rad Laboratories, Inc.). RNA extracts with an A260/A280 ratio between 1.8-2.0 and three complete bands of 28S, 18S and 5S, after 2% agarose gel electrophoresis, were chosen for reverse transcription (Fig. 1A and B). A cDNA Reverse Transcription kit (Fuji Biological Co., Ltd.) was used to perform the reverse transcription procedure using the following reverse transcription temperature

Table I. Clinical characteristics.

Category	Severe preeclampsia	Normotensive pregnancy	t	P-value
Maternal age, years	32.2±5.226	30.40±5.475	0.9211	0.365
Gestational age, weeks	33.18±1.102	38.78±1.015	3.439	<0.001[a]
Maximum systolic pressure, mmHg	172.0±8.577	112.7±8.932	18.54	<0.001[a]
Maximum diastolic pressure, mmHg	117.2±8.711	72.93±7.887	11.29	<0.001[a]

[a]P<0.01. Data are presented as the mean ± SD.

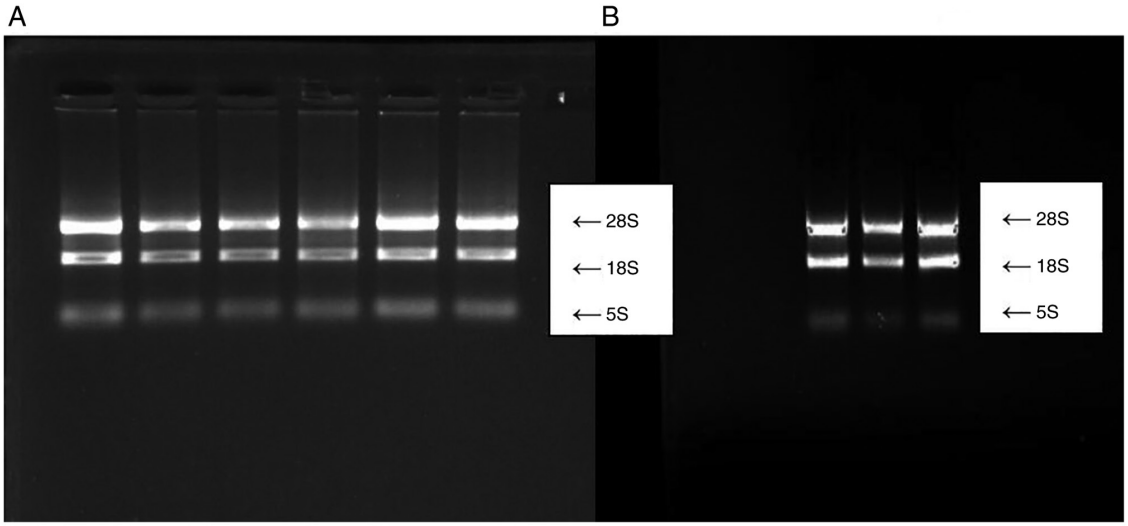

Figure 1. RNA agarose gel electrophoresis. Images of complete RNA extracted from (A) the placental artery and (B) HUVECs showing three bands corresponding to ribosomal subunits 28S, 18S and 5S, respectively.

protocol: 42˚C for 15 min, 85˚C for 5 sec and cooling at 4˚C. A SYBR Green MasterMix kit (Toyobo Co., Ltd.) was used for qPCR in a StepOnePlus PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.), in a 20-μl reaction system with the following amplification temperature protocol: 95˚C for 10 min, followed by 40 cycles of 95˚C for 5 sec, 58˚C for 20 sec and 72˚C for 10 sec. GAPDH was used as the internal reference, and the internal reference and target gene primers were designed and synthesized by Shanghai Sangon Biological Co., Ltd. (Table II). Finally, the relative expression of each target gene was calculated using the $2^{-\Delta\Delta Cq}$ method (17). The experiments were repeated three times.

Western blot analysis. Total protein was extracted from tissues and cells using RIPA lysis buffer (Biyuntian Biotechnology Co., Ltd.) containing the protease inhibitor phenylmethanesulfonylfluoride (Biyuntian Biotechnology Co., Ltd.), and protein concentration was measured using a BCA protein assay kit (Biyuntian Biotechnology Co., Ltd.). The protein samples were denatured, and 25 μg protein per sample was loaded per lane on a 10% SDS gel, resolved using SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked in 5% skimmed milk powder dissolved in 0.5% Tween-TBS for 2 h at room temperature. After washing the membranes, they were incubated overnight at 4˚C on an agitator with one of the following primary antibodies: Mouse anti-GAPDH (1:10,000; cat. no. MB001H; Bioworld), rabbit anti-TRPV1 (1:1,000; cat. no. KL-KT-008292; Abcam), rabbit anti-ABCC9 (also known as SUR2B; 1:500; cat. no. ab84299; Abcam), rabbit anti-Kir6.1 (1:1,000; cat. no. TF6468R; Crystal Wind Biological Co., Ltd.) or rabbit anti-eNOS (1:1,000; cat. no. ab15280-eNOS; Abcam). The following day, after the membranes were washed, the secondary HRP-conjugated Goat Anti-Rabbit IgG antibody (1:1,000; cat. no. PF01924; Pufei Bio Co., Ltd.) was added and incubated at 37˚C for 2 h. Densitometry analysis was performed using ImageJ version 1.49 (National Institutes of Health). Experiments were repeated three times.

Immunohistochemistry. Immunohistochemistry was performed using an HRP Streptavidin Conjugate kit according to the manufacturer's protocol (DBA cat. no. Im-05818B; Shanghai Caiyou Industrial Co., Ltd.). After baking at 60˚C for 2 h, the paraffin sections were dewaxed and hydrated in turn, washed with PBS and incubated with 3% hydrogen peroxide (TITABIO; cat. no. SY2622; Beijing Ita Biological Co., Ltd.) for 5-10 min at room temperature to quench endogenous peroxidase activity. The paraffin sections were subjected to antigen retrieval in a microwave in EDTA buffer (cat. no. AS1016; Wuhan Aspen Biotechnology Co., Ltd.). Subsequently, blocking solution (solution B included in the kit) was added and samples were incubated

Table II. Primer sequences.

Gene	Primer sequence	PCR product size, bp
GAPDH forward	5'-CCACTCCTCCACCTTTG-3'	106
GAPDH reverse	5'-CACCACCCTGTTGCTGT-3'	106
TRPV1 forward	5'-CATCATCCTGCTGGCCTATG-3'	105
TRPV1 reverse	5'-TTGCTCTGTGCGATCTTGTTG-3'	105
Kir6.1 forward	5'-GATCATCTGCCACGTGATTGA-3'	150
Kir6.1	5'-GCAATGTAGGAGGTTCGTGCT-3'	150
SUR2B forward	5'-CGGGACATAACCTGAGATGG-3'	130
SUR2B reverse	5'-ATCACGGCTGGCATAAAGAG-3'	130
eNOS forward	5'-GTGGCTGGTACATGAGCACT -3'	180
eNOS reverse	5'-TGGCTAGCTGGTAACTGTGC-3'	180

eNOS, endothelial nitric oxide synthase; TRPV1, transient receptor potential cation channel subfamily V member 1.

at room temperature for 30 min. The primary antibody working solution containing anti-TRPV1 (1:200 dilution; cat. no. KL-KT-008292; Abcam), anti-ABCC9 (1:200 dilution; cat. no. ab84299; Abcam), anti-Kir6.1 (1:200 dilution; cat. no. TF6468R; Crystal Wind Biological Co., Ltd.) or anti-eNOS (1:2,000 dilution; cat. no. ab15280-eNOS; Abcam) was added to cover the sections, which were incubated at 4˚C overnight. The following day, the samples were rinsed, and to stain the sections, the streptavidin-biotin-peroxidase complex was used after the sections had been treated with biotinylated goat anti-rabbit immunoglobulin G secondary antibody for 30 min at room temperature. A DBA solution was used to develop the stain. Next, the samples were stained again with hematoxylin for 5 min at room temperature and differentiated by hydrochloric acid alcohol, dehydrated, made transparent and sealed with neutral glue. Finally, the samples were observed and imaged under a light microscope (400x magnification; Leica Aperio AT2; Leica Microsystems, Co., Ltd.). The expression levels of various proteins in the endothelial cells were observed. Brown-yellow staining indicated positive expression, and the optical density value represented the relative expression level. The images were analyzed using Q-IMAGING software (MicroPublisher 6 CCD; Nano Hai Bioscientific Instruments Co., Ltd.).

Measurement of total nitrite levels. An NO assay kit (Nanjing Jiancheng Biological Co., Ltd.) was used in the present study according to the manufacturer's protocol. Prior to the experiment, solutions III, IV and V were prepared into chromogenic agents according to the ratio of 2.5:1:1. A total of 300 μl homogenate supernatant and 100 μl plasma was deproteinized by adding 200 μl reagent I and stirring, after which 100 μl reagent II was added, the solution was stirred and centrifuged at 3,500-4,500 x g for 15 min at 4˚C, and the supernatant was recovered. A preheated microplate reader (BMG LABTECH, CLARIOstar PLUS) (preheated for >30 min) was used and the wavelength was adjusted to 550 nm. The samples to be tested were added to the 96-well plate, mixed, and left for 15 min, after which the OD value of absorbance in each well was measured using a microplate reader.

Statistical analysis. All data were analyzed using SPSS version 20.0 (IBM Corp.) and GraphPad Prism 5.0 (GraphPad Software, Inc.). Normally distributed data are presented as the mean ± SD. An independent samples t-test was used to compare the NP and SP groups, and a one-way ANOVA followed by a Newman-Keuls multiple comparison post-hoc test was used for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient demographics and clinical characteristics. Table I presents the demographic and clinical characteristics of the included patients when they were admitted to hospital, including maternal age, gestational age and blood pressure. There was no significant difference in maternal age between the NP and SP groups ($P>0.05$), but the gestational age difference was statistically significant in the two groups ($P<0.01$). In addition, systolic and diastolic blood pressure were significantly higher in the SP group than in the NP group ($P<0.01$).

Endothelial layers are impaired in severe preeclampsia. As shown in Fig. 2A endothelial cells in the NP group were uniformly and regularly arranged, and the nucleus was oblong, bluish in color and protruded slightly from the official cavity. In the SP group, the walls of the placental artery were thickened, the number of collagen fibers had increased, the artery was hyalinized, the endothelial cells were arranged in a disorderly manner and the endodermis was notably damaged (Fig. 2B).

TRPV1, Kir6.1/SUR2B and eNOS expression levels are decreased in the placental artery of patients with severe preeclampsia. Immunohistochemistry was used to evaluate the localization of the TRPV1, Kir6.1/SUR2B and eNOS channels in the placental artery (Figs. 3 and 4). The results showed that each channel was primarily distributed in the vascular endothelial layer. It was noted that the number of counterstained nuclei in the endothelial cell layer was significantly decreased, as was the membrane staining of each channel in the SP group compared with the NP group. The OD values of TRPV1, Kir6.1/SUR2B and eNOS in the SP

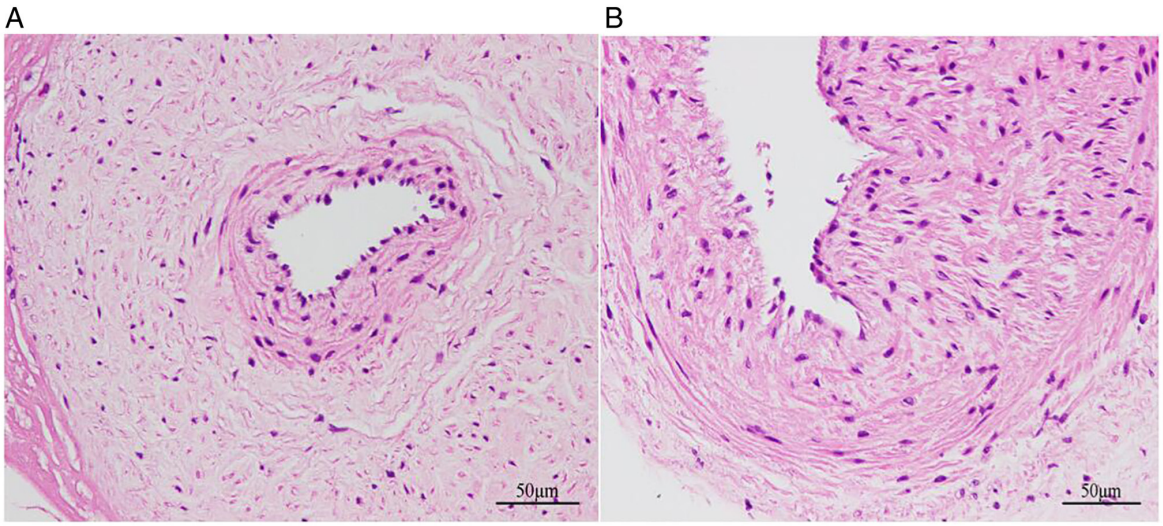

Figure 2. Hematoxylin and eosin staining of the placental artery in the SP group and NP group. The endothelial cells of the placental artery were intact in the (A) NP group, and the endodermis of the placental artery was significantly damaged in the (B) SP group. Magnification, x400. SP, severe preeclampsia; NP, normotensive pregnancy.

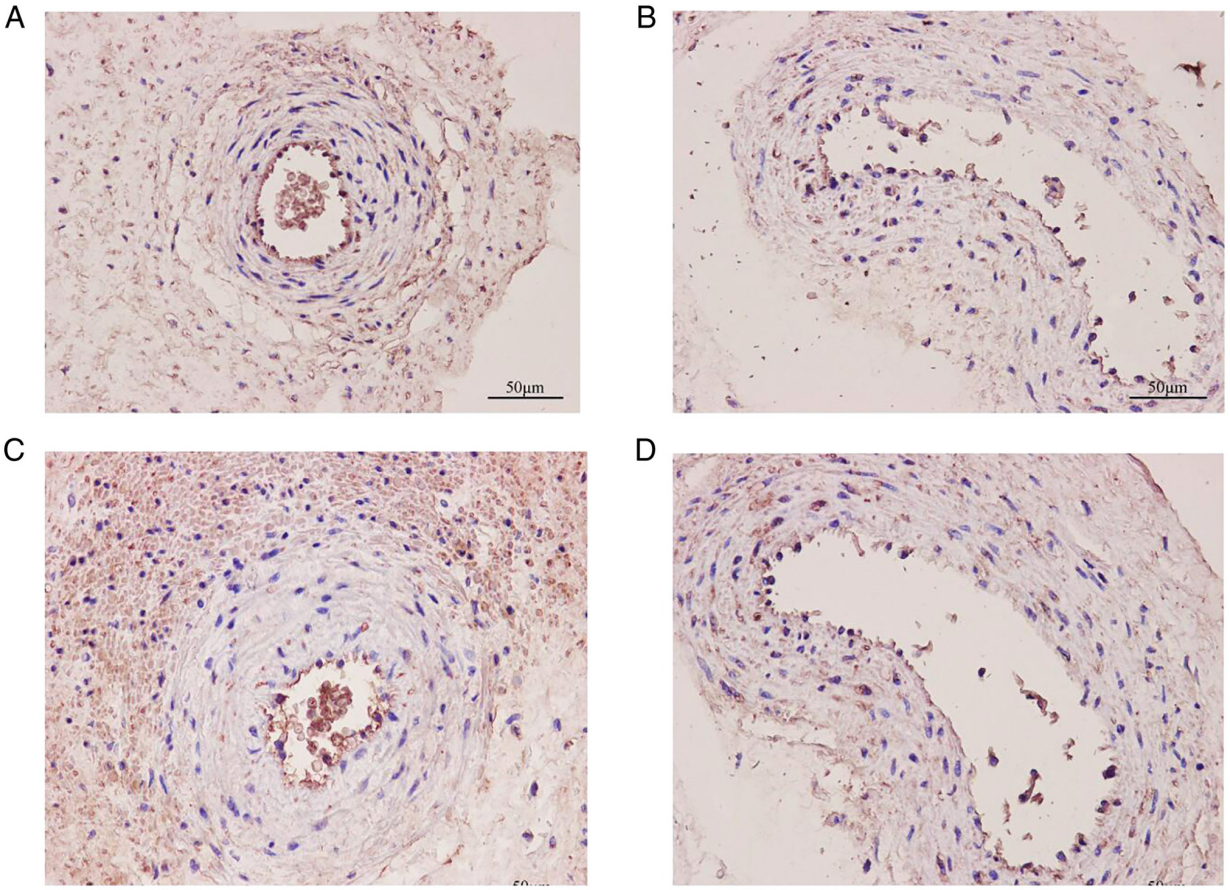

Figure 3. Immunohistochemical analysis of TRPV1 and Kir6.1 expression in the two groups. TRPV1 and Kir6.1 channels are expressed in the SP and NP groups, and are primarily distributed in the vascular endothelial layer: (A) TRPV1 in the SP group, (B) TRPV1 in the NP group, (C) Kir6.1 in the SP group and (D) TRPV1 in the NP group. Magnification, x400. SP, severe preeclampsia; NP, normotensive pregnancy; TRPV1, transient receptor potential cation channel subfamily V member 1.

group were 11,660.0±1,721.0, 2,975.0±505.5, 10,236.0±1,355.0 and 14,663.0±2,320.0, respectively, which were lower than those in the NP group (24,917.0±2,044.0, 4,495.0±775.0, 14,663.0±2,320.0, and 20,988.0±2,289.0; all P<0.05) (Fig. 5). RT-qPCR was used to examine the relative expression of the four-channel genes. The relative expression of TRPV1, Kir6.1, SUR2B and eNOS in the SP group was 0.559±0.609, 0.419±0.281, 0.166±0.087 and 0.383±0.110, respectively,

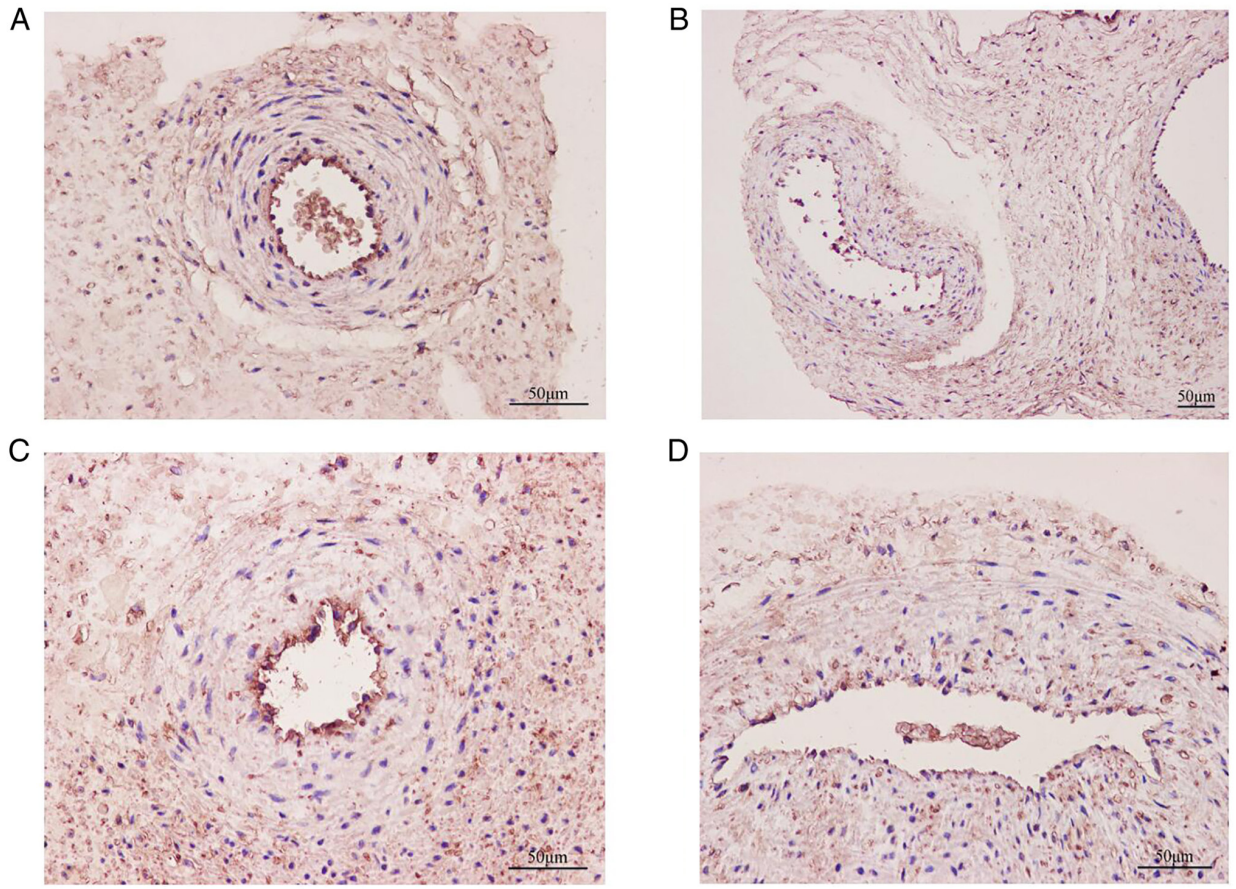

Figure 4. Immunohistochemical analysis of SUR2B and eNOS in the SP and NP groups. SUR2B and eNOS channel are expressed in the two groups, and are primarily distributed in the vascular endothelial layer: (A) SUR2B in the SP group, (B) SUR2B in the NP group, (C) eNOS in the SP group and (D) eNOS in the NP group. Magnification, x400. SP, severe preeclampsia; NP, normotensive pregnancy; eNOS, endothelial nitric oxide synthase; TRPV1, transient receptor potential cation channel subfamily V member 1.

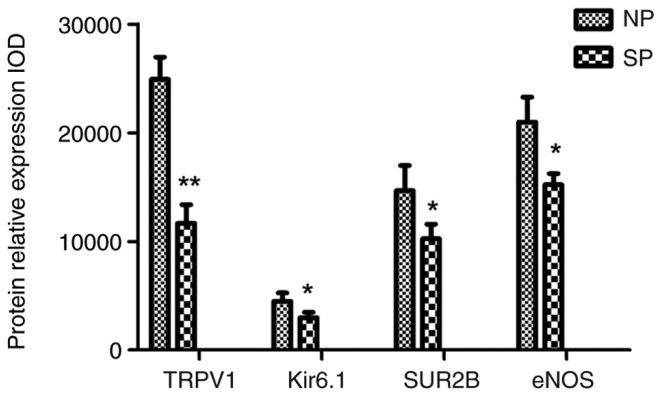

Figure 5. Histogram of the optical density of TRPV1, Kir6.1/SUR2B and eNOS expression in the two groups based on immunohistochemistry. TRPV1, Kir6.1/ SUR2B and eNOS levels in the SP group were significantly lower than those in the NP group. *P<0.05 and **P<0.01 vs. NP. SP, severe preeclampsia; NP, normotensive pregnancy; eNOS, endothelial nitric oxide synthase. SP, severe preeclampsia; NP, normotensive pregnancy; eNOS, endothelial nitric oxide synthase; TRPV1, transient receptor potential cation channel subfamily V member 1; IOD, integrated optical density.

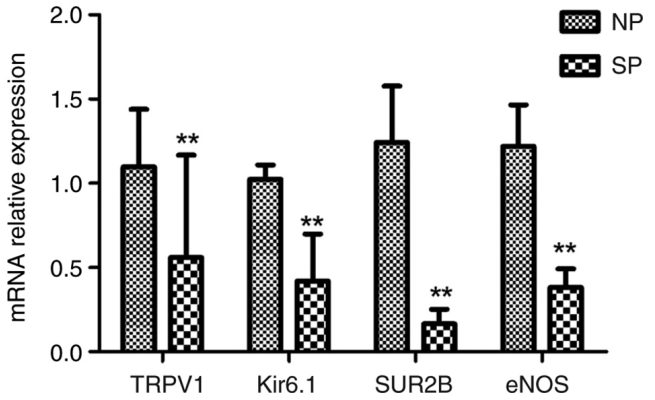

Figure 6. Histogram of relative gene expression of TRPV1, Kir6.1/ SUR2B and eNOS in the two groups. TRPV1, Kir6.1/SUR2B and eNOS levels in the SP group were significantly lower than those in the NP group. **P<0.01 vs. NP. SP, severe preeclampsia; NP, normotensive pregnancy; eNOS, endothelial nitric oxide synthase; TRPV1, transient receptor potential cation channel subfamily V member 1.

which was significantly lower than that in the NP group (1.098±0.341, 1.024±0.085, 1.243±0.335 and 1.219±0.247; all P<0.01) (Fig. 6). Since gene expression does not fully reflect protein expression, western blotting was used to quantitatively examine whether this difference was also present at the protein level (Fig. 7). The relative quantitative analysis results showed that the ratio of the gray value of each target protein to GAPDH in the SP group was 0.282±0.058, 0.058±0.005, 0.132±0.007 and 0.059±0.023, respectively, which was lower than that in

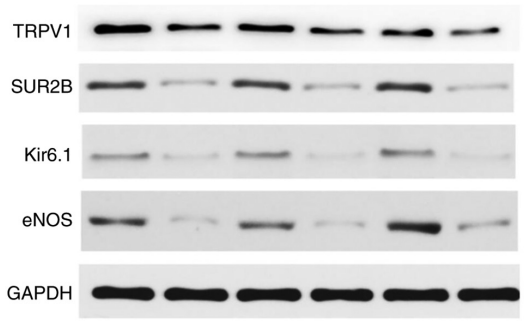

Figure 7. Comparison of the relative protein expression levels of TRPV1, Kir6.1/SUR2B and eNOS in the two groups. SP, severe preeclampsia; NP, normotensive pregnancy; eNOS, endothelial nitric oxide synthase; TRPV1, transient receptor potential cation channel subfamily V member 1.

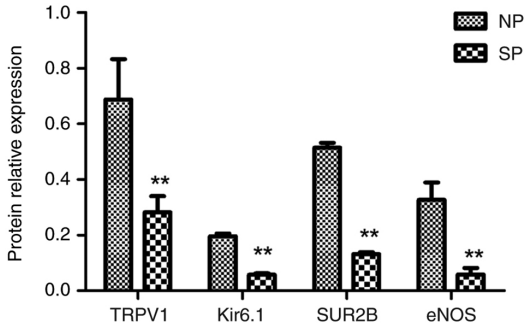

Figure 8. Histogram of the relative protein expression of TRPV1, Kir6.1/SUR2B and eNOS in the two groups. TRPV1, Kir6.1/ SUR2B and eNOS expression levels in the SP group were significantly lower than those in the NP group. **P<0.01 vs. NP. SP, severe preeclampsia; NP, normotensive pregnancy; eNOS, endothelial nitric oxide synthase; TRPV1, transient receptor potential cation channel subfamily V member 1.

the NP group (0.688±0.145, 0.196±0.010, 0.514±0.018 and 0.327±0.063; all P<0.01) (Fig. 8), suggesting that the relative protein expression of TRPV1, KATP subtype Kir6.1/SUR2B and eNOS in the SP group was also significantly downregulated.

Roles of eNOS and Kir6.1/SUR2B channels in capsaicin/capsazepine-induced relaxation in HUVECs. To explore the interactions of the four channels, further cytological experiments were performed. The relative gene expression of TRPV1, Kir6.1, SUR2B and eNOS in the control group was 0.986±0.129, 1.439±0.358, 1.479±0.403 and 1.162±0.090, respectively, while in the capsaicin group, it was 4.568±0.810, 4.014±0.781, 5.505±0.287 and 2.821±0.377, respectively, and in the capsazepine group, it was 0.077±0.010, 0.036±0.014, 0.046±0.010 and 0.083±0.005, respectively; the differences between the control group and capsaicin groups, and the capsazepine group were statistically significant (all P<0.01; Fig. 9). The results of western blotting showed that the relative protein expression of TRPV1, Kir6.1, SUR2B and eNOS in the control group was 0.266±0.026, 0.226±0.014, 0.166±0.013 and 0.277±0.025, respectively, while the expression in the capsaicin group was 0.763±0.044, 0.687±0.046, 0.493±0.035 and 0.498±0.022, respectively, and expression in the capsazepine group was 0.157±0.040, 0.057±0.005, 0.065±0.005

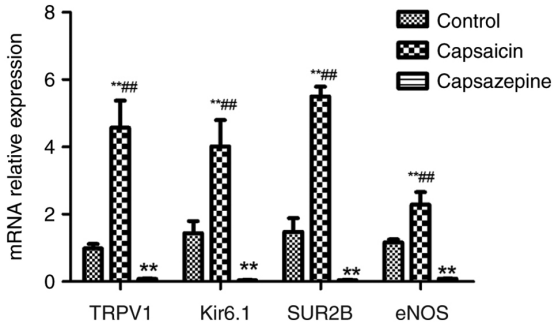

Figure 9. Relative gene expression of TRPV1, Kir6.1/SUR2B and eNOS in cells treated with capsaicin or capsazepine. TRPV1, Kir6.1/SUR2B and eNOS expression in the control group was lower than that in the capsaicin group (1 μM), but higher than that in the capsazepine group (1 μM) **P<0.01 vs. control; ##P<0.01 vs. capsazepine. SP, severe preeclampsia; NP, normotensive pregnancy; eNOS, endothelial nitric oxide synthase; TRPV1, transient receptor potential cation channel subfamily V member 1.

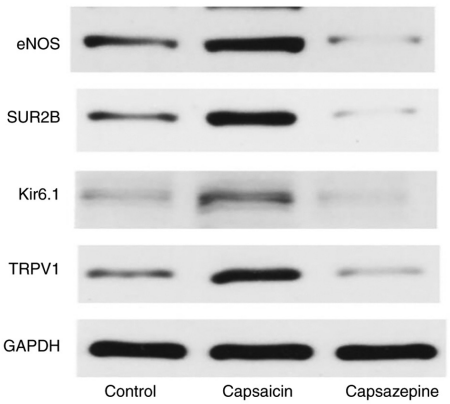

Figure 10. Relative protein expression of TRPV1, Kir6.1/SUR2B and eNOS in cells treated with capsaicin or capsazepine. eNOS, endothelial nitric oxide synthase; TRPV1, transient receptor potential cation channel subfamily V member 1.

and 0.058±0.008, respectively (all P<0.01), and these differences between the control group and capsaicin groups, and the capsazepine group were statistically significant (P<0.01; Figs. 10 and 11).

NO levels are decreased in the placental artery and serum of patients with severe preeclampsia. The nitrate reduction method indicated that the mean levels of total nitrites in the placental artery and plasma were 33.31±11.73 and 45.12±14.92, respectively, in severe preeclampsia patients, which were significantly lower than those in the control group (47.65±9.81 and 66.01±13.81, respectively; both P<0.01) (Figs. 12 and 13).

Discussion

The vascular endothelium is present throughout the body; it acts not only as a mechanical barrier between blood and smooth muscle cells, but also as the body's largest endocrine organ, which can synthesize and release various vasoactive substances through multiple mechanisms to regulate vascular tension (18-20). It is generally accepted that endothelial dysfunction promotes the occurrence of cardiovascular

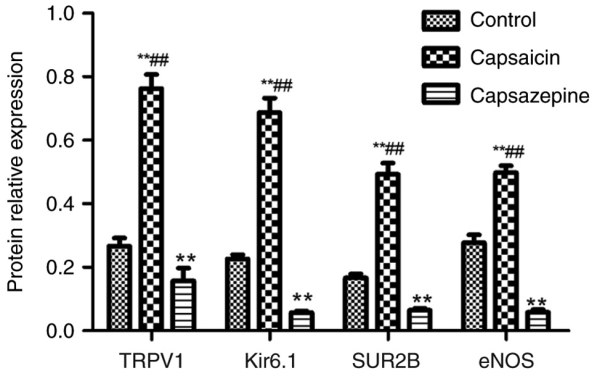

Figure 11. Histogram of the relative protein expression of TRPV1, Kir6.1/SUR2B and eNOS in cells treated with capsaicin or capsazepine. TRPV1, Kir6.1/SUR2B and eNOS expression in the control group was lower than that in the capsaicin group (1 μM), but higher than that in the capsazepine group (1 μM) **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. capsazepine. SP, severe preeclampsia; NP, normotensive pregnancy; eNOS, endothelial nitric oxide synthase; TRPV1, transient receptor potential cation channel subfamily V member 1.

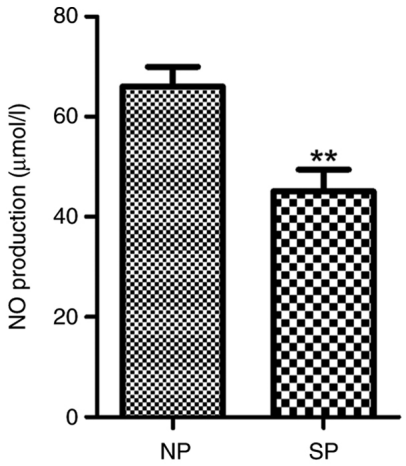

Figure 12. Histogram of the mean serum NO levels in the NP and SP groups. Total plasma nitrite levels in the SP group were significantly lower than those in the NP group. **P<0.01 vs. NP. SP, severe preeclampsia; NP, normotensive pregnancy; NO, nitric oxide.

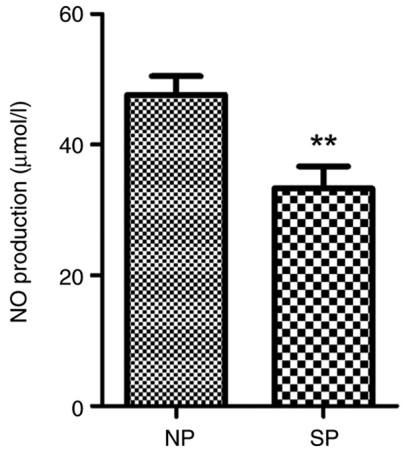

Figure 13. Histogram of the mean NO levels in the placental artery lysates in the NP and SP groups. Total plasma nitrite levels in the SP group were significantly lower than those in the NP group. **P<0.01 vs. NP. SP, severe preeclampsia; NP, normotensive pregnancy; NO, nitric oxide.

diseases, such as hypertension, and that the degree of endothelial injury is directly related to the severity of hypertension. In patients with hypertension, endothelial cells are damaged due to elevated blood pressure, which reduces the secretion of NO and other vasoactive substances, thus leading to endothelial-dependent diastolic dysfunction, which in turn becomes an initiating factor of various complications of hypertension, forming a vicious cycle (21). As the leading cause of fetal growth retardation and infant morbidity and mortality associated with premature delivery and maternal death, studies have shown that preeclampsia is a complex obstetric syndrome associated with maternal vascular dysfunction in which the NO signaling pathway is a crucial driver of disease progression and severity (22). It is common knowledge that the placental vasculature plays a crucial role in the placenta's ability to function normally. The substantial placental vascularization and the placenta's sophisticated processes that control vascular tone assist the fetus's growing needs for blood supply as the pregnancy progresses (23). NO in the syncytiotrophoblast and endothelial cells of the chorionic plate and stem villous vessels is suggested to cause the dilatation of the human placental vasculature (24). The results of the present study showed that in individuals with severe preeclampsia, the vascular walls of the placental artery were thickened, the presence of collagen fibers was increased, the artery hyaline was altered, and the endothelial cell layer was notably damaged. Dikensoy *et al* (25) found that the mean serum levels of NO were significantly lower in patients with severe or moderate preeclampsia compared to control subjects, and this decrease was correlated with the severity of the disease. Similar to these findings, in the present study, a chemical reagent was used to determine the amount of NO in the mother's serum and in the placental artery. It was shown that there were functional alterations following vascular endothelial injury in patients with severe preeclampsia, as the NO levels in the patients with severe preeclampsia were much lower than those in the control group, both in the serum and placental tissue lysates. In fact, numerous studies have demonstrated the role of NO in the regulation of fetal-maternal vascular tone in both animals and humans (25-27). It was also found that infusion of eNOS inhibitor in mice during pregnancy resulted in hypertension and fetal growth retardation, similar to that observed in patients with preeclampsia (26). Boeldt and Bird (28) showed that maternal peripheral endothelial dysfunction is central to the disease stage of preeclampsia. However, it is still unclear how diminished placental perfusion causes severe maternal vascular endothelial dysfunction. The majority of *in vitro* studies claim that Ca^{2+}-dependent eNOS is the most prevalent NOS isoform in the human placenta and is responsible for placental NO generation (29). The findings of the present study revealed that the relative expression of eNOS was notably downregulated in patients with severe preeclampsia compared with that in women with normal-term pregnancies. Thus, we hypothesize that normal placenta formation may require locally generated NO to promote intravascular infiltration of cytotrophoblast cells. The decreased expression of eNOS in the placental artery results in a decrease in locally generated NO synthesis, leading to the preeclampsia-related impairment of cytotrophoblast invasion, diastolic endothelial dysfunction and vasospasm, which form a vicious cycle and participate in

the changes in uterine placental ischemia and hypoxia, and the release of a variety of placental factors, which enter maternal blood circulation, activate systemic inflammatory reaction, damage vascular endothelial cells, and eventually lead to hypertension and various complications, consistent with the classical theory of preeclampsia due to poor placentation (30).

TRPV1 was originally discovered by researchers to be specifically activated by capsaicin, which is why it is also known as the capsaicin receptor (31). As the most studied member of the TRPV subfamily, TRPV1 was originally identified in the nervous system and is present in the vagus nerve, trigeminal ganglion and dorsal ganglion neurons (32). In recent years, an increasing number of studies have shown that TRPV1 also plays an important role in the regulation of cardiovascular disease and is primarily expressed in cardiomyocytes, smooth muscle cells, vascular endothelial cells, inflammatory cells and peripheral vascular adipose tissue (33-36). TRPV1 is a non-selective cationic channel that can mediate the transmembrane flow of cations, dominated by Ca^{2+}, when ligands bind to the receptor, which can result in changes in intracellular Ca^{2+} concentrations and activate a series of intracellular signals to participate in a variety of intracellular physiological and pathological processes (33,37). The synthesis of NO is closely related to the increase in intracellular Ca^{2+}. Ca^{2+} binding to calmodulin in endothelial cells can activate eNOS and promote the synthesis and release of NO, thereby dilating blood vessels and decreasing vascular resistance (38-40). TRPV1 may play an essential role in endothelial cell physiological and pathological status to allow the maintenance of normal vascular function and the pathological formation of vascular lesions. It has been confirmed in animal experiments that TRPV1 can mediate coronary artery relaxation in an endothelium-dependent manner (4), and TRPV1 can stimulate NO synthesis through different signaling pathways (6,41). It is well established that the structural dysfunction of potassium channels can disrupt the balance of vasoconstriction and the diastole of blood vessels themselves, increasing vascular tension and eventually leading to the pathological state of hypertension (42,43). KATP is a potassium ion channel; reports on KATP have primarily focused on vascular smooth muscle cells, and researchers later showed that KATP in endothelial cells also participates in regulating vascular tone (44). Studies have shown that the KATP agonist cromakalim can increase Ca^{2+} levels in endothelial cells, which can increase the expression of eNOS, promote the synthesis and release of NO, and indirectly relax smooth muscle tissue (8,9). Bratz *et al* (4) found that endothelium-dependent dilation mediated by TRPV1 could be attenuated by iberiotoxin, a selective inhibitor of Ca^{2+}-activated K^+ channels (BKca), suggesting that BKca is involved in capsaicin-induced relaxation. In addition, tetraethylammonium, a non-specific potassium channel blocker, also attenuates TRPV1-mediated endothelium-dependent relaxation, suggesting that multiple potassium channels are involved in TRPV1-mediated endothelium-dependent relaxation. The present study showed that TRPV1 and Kir6.1/SUR2B are primarily expressed in the endothelial cell layer in human placental arteriolar cells, and the relative gene and protein expression of TRPV1 and Kir6.1/SUR2B in the experimental group is significantly downregulated compared with that in the control group. Thus, we hypothesize that there might be some correlation between TRPV1 and the KATP. Chen *et al* (45) conducted animal experiments on Wistar rats and showed that capsazepine, a selective TRPV1 blocker could be used to prevent endothelium-dependent dilatation and enhance NO release induced by Natakalim, a novel KATP opener in the mesenteric arterioles of rats. To further characterize the signaling cascade behind the TRPV1-KATP mediated relaxation, in the present study, HUVECs were used and it was shown that in the agonist and blocker groups, the relative expression of SUR2B/Kir6.1 and eNOS was also significantly upregulated and downregulated, respectively, compared with that in the control group, indicating that the activation or inhibition of TRPV1 could upregulate or downregulate the expression of Kir6.1/SUR2B and eNOS, which suggests that the TRPV1-specific agonist capsaicin is working via the KATP channel as a result of a second messenger, most notably NO. Referring to the previous theory, we hypothesize that TRPV1 activation in endothelial cells activates Kir6.1/SUR2B via an unknown mechanism, causing hyperdifferentiation of the endothelial membrane and thereby boosting Ca^{2+} influx into cells. As a non-selective cation channel, TRPV1 can also mediate the transmembrane flow of cations dominated by Ca^{2+} when the ligand binds to its receptor. Through the aforementioned two possible mechanisms, Ca^{2+} levels can be further elevated in endothelial cells, enhancing the expression and activity of eNOS, thereby increasing the synthesis and release of NO, and the endothelium-dependent vasodilation response. Breyne and Vanheel (46) demonstrated that stimulation of TRPV1 may lead to the release of calcitonin gene-related peptides, which hyperpolarize the smooth muscle cells in rat mesenteric arteries by activating KATP channels. However, the present study found that TRPV1 and KATP channels were primarily distributed in the endothelial cell layer in the placental artery, which may be caused by species differences. We hypothesize that TRPV1 channels are functionally expressed in the placental artery and mediate endothelium-dependent vasodilation through a mechanism involving NO and KATP channels. In patients with severe preeclampsia, the expression of TRPV1 and Kir6.1/SUR2B is decreased due to endothelial cell damage, and the TRPV1-KATP channel-mediated vasodilation is also impaired, leading to endothelial dysfunction. In addition, NO diffusion to the vascular smooth muscle is correspondingly decreased, and vascular smooth muscle cannot relax properly, resulting in decreased placental perfusion and secondary systemic circulatory disturbances, which underlie the clinical symptoms of severe preeclampsia. Animal models are vital to determine the mechanism of action of TRPV1/KATP *in vivo*. The ideal animal experiment would be to place an experimental group of animals on a capsaicin diet (TRPV1-specific agonist) and ultimately observe the blood pressure, the size and weight of the placenta, the birth weight and survival of the pups, and the levels of certain biochemical markers in the mother. Researchers have found that infusion of an NO synthesis inhibitor in mice during pregnancy results in hypertension and fetal growth retardation, which are similar to the signs of preeclampsia. However, severe preeclampsia is a spontaneous disease of pregnant women with certain risk factors after 20 weeks of gestation, while other animals do not develop preeclampsia and the gestational age of animals is difficult to match with that of humans; thus, there is no well-recognized animal model as of yet. Due to the limitations of experimental conditions, it was not possible to conduct vascular ring tension tests, additional

electrophysiological experiments or animal experiments, which are significant limitations of this study and will thus serve as a direction for future studies.

In conclusion, as a specific type of hypertensive disease during pregnancy, severe preeclampsia may be characterized by multiple factors, mechanisms and pathways. The present study showed that the impairment of the endothelial TRPV1-KATP channel-mediated eNOS/NO pathway may play an important role. However, further vascular ring tension tests, additional electrophysiological experiments and animal experiments are still necessary to further understand the etiology and pathogenesis of this disease, and to provide a novel theoretical basis for its prevention, diagnosis and treatment.

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Availability of data and materials

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

Authors' contributions

XZ and XF conceived and designed the study. XZ, LW, HL and YT performed the experiments and statistical analyses. XZ and XF wrote the manuscript. HL, YT and XF reviewed and edited the manuscript. All authors have read and approved the final manuscript. XZ and HL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

This study was approved by the Clinical Trial Ethics Committee of the Affiliated Hospital of Southwest Medical University (approval no. KY2019039). All work was undertaken according to the provisions described in the Declaration of Helsinki and its later amendments. All patients signed informed consent for specimen collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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