

Original Article

TRPV6 channel expression is associated with the proliferation of trophoblasts and pathogenesis of early-onset preeclampsia

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Abstract: Although preeclampsia is a major cause of maternal morbidity and mortality, the pathogenesis remains unclear. TRPV6 plays an important role in maternal-fetal Ca^{2+} transport. A total of 30 pregnant women were included and assigned to two groups: early-onset PE pregnancies (n = 15) and late-onset PE pregnancies (n = 15). Fifteen normal pregnant women comprised a control group. The levels of TRPV6 mRNA and protein expression in the placentas of the women were measured by RT-PCR and immunohistochemistry. HTR-8/SVneo cells (trophoblast cells present at the early stage of pregnancy) were cultured in vitro, and the effects of a TRPV6 channel blocker (ruthenium red) on the proliferation and Ca^{2+} influx of HTR-8/SVneo cells were determined using the MTT assay and a calcium indicator- Fluo-4 AM. The levels of TRPV6 mRNA and protein expression in the placentas of women with early-onset PE were significantly lower when compared with those expression levels among women in the control group and late-onset PE group. There were no significant differences in the levels of TRPV6 mRNA and protein expression between women in the late-onset PE group and control group. Ruthenium red inhibited the proliferation of HTR-8/SVneo cells in a time-dependent manner and reduced the level of Ca^{2+} influx. The results show that TRPV6 gene was reduced in early-onset preeclampsia placental tissue. And TRPV6 probably participates in the pathogenesis of preeclampsia by influencing the proliferation of trophoblast cells.

Keywords: TRPV6, preeclampsia, HTR-8/SVneo cells, cell proliferation

Introduction

Preeclampsia (PE) is a pregnancy-induced hypertensive disorder with incidence rates ranging from 3% to 7% among nulliparas females and 1% to 3% among multiparas females [1]. PE is a major cause of maternal morbidity and mortality worldwide [2], and 10%-15% of all maternal deaths are directly associated with preeclampsia or eclampsia [3]. PE is characterized by development of a maternal syndrome that includes hypertension, coagulation abnormalities, edema, proteinuria, and vascular abnormalities. These symptoms usually develop after 20 weeks of gestation and disappear within 7 to 10 days after delivery.

During the late 1970s and early 1980s, several articles were published which described preeclampsia as two distinct disease entities: (1) early-onset preeclampsia, which tends to de-

velop before 34 weeks of gestation, and (2) late-onset preeclampsia, which develops at or after 34 weeks of gestation. Since then, several additional articles have been published which support this distinction [4]. A large prospective study recently reported a 0.46% prevalence of early-onset PE compared to a 1.6% prevalence of late-onset PE among pregnant women. Despite its lower frequency, early-onset PE is of paramount importance to medical practitioners because of its strong association with adverse perinatal outcomes [5]. However, the precise factors involved in the pathogenesis of PE remain unclear, and it continues to be considered a multisystem disorder. Two well-known pathogenic features of preeclampsia include placental and vascular abnormalities. Shallow trophoblast invasion and improper remodeling of spiral arteries are among the best recognized and most frequently reported physiologic features of PE [6, 7].

Transient receptor potential (TRP) channels constitute a large family of nonselective ion channel proteins which play key roles in a variety of physiological functions [8]. TRPV6 (transient receptor potential vanilloid 6) is one of the most highly calcium selective channels, and when compared with the other TRP channels, is characterized by its high selectivity for calcium and active role in Ca^{2+} -related intracellular pathways [9, 10]. Several studies have demonstrated over-expression of TRPV6 in several types of cancer, including prostate [11, 12], breast [13-15], and colon cancer [16], and shown that TRPV6 promotes tumor invasion and metastasis via a Ca^{2+} -related mechanism.

Ca^{2+} is a universal intracellular second messenger involved in numerous biological processes, including signal transduction, neurotransmission, enzyme and hormone secretion, cell cycle regulation, and programmed cell death. Additionally, it is crucial for adequate fetal development and prenatal programming of future diseases. TRPV6 mRNA is highly expressed in the placentas of humans and mice, and plays an important role in maternal-fetal Ca^{2+} transport [17-21]. The Ca^{2+} concentrations in the fetal blood and amniotic fluid of murine TRPV6 knockout (KO) fetuses are significantly lower than those in wild type fetuses; however, the physiological role of placental TRPV6 remains unknown. A recent study showed that samples of placental tissue obtained from women with preeclampsia contained lower levels of TRPV6 protein when compared with samples of healthy placental tissue [22].

Histopathological examinations of placental tissue obtained from women with pre-eclampsia have revealed different morphological characteristics depending on the timing of disease onset. However, it is unknown whether such differences reflect differential placental gene expression. Therefore, we conducted the current study to examine TRPV6 expression in the placental tissue of women with early-onset PE and late-onset PE, and examine the role played by TRPV6 in the proliferation of trophoblast cells.

Materials and methods

Patients and tissue specimens

Samples of human placental tissue were obtained in accordance with guidelines estab-

lished by the Ethics Committee of Qingdao Municipal Hospital, China. The placentas were obtained immediately after cesarean delivery of term pregnancies (n = 15), early-onset PE pregnancies (n = 15), and late-onset PE pregnancies (n = 15). PE was defined as a systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg as recorded on two occasions separated by at least four hours, and occurring after 20 weeks of gestation in a pregnant woman with previously normal blood pressure and detectable proteinuria (≥ 0.3 g/24 hrs). All women enrolled in the study lacked any pre-existing clinical risk factors for gestational hypertension. Women with the following conditions were excluded from the study: pre-pregnancy diabetes mellitus, gestational diabetes, foetal physical or chromosomal abnormalities, untreated hypo/hyperthyroidism, renal diseases with altered renal function, active and chronic liver disease, cancer, collagen diseases, congenital rickets and other malformations. The control group consisted of 15 women without a diagnosed pathology. All samples of placental tissue were snap frozen in liquid nitrogen and stored at -280°C until analysis.

RT-PCR

RNA was isolated from 30 mg samples of total placental tissue using RNeasy mini kit extraction columns (Qiagen; Hilden, Germany), and then reverse-transcribed. The extracted mRNA was transcribed into first strand cDNA using oligo-dT primers and M-MLV reverse transcriptase. Specifically designed PCR primers were used to amplify the RT-generated TRPV6 cDNAs molecules. The sense primer for TRPV6 was 5'ATGGTGATGCGGCTCATCAGTG3', and the antisense primer was 5'GTAGAAGTGGCCTAGC-TCCTCG3'. The sense primer for GAPDH was 5'ACCACAGTCCATGCCATCAC3', and the antisense primer was 5'TCCACCACCCTGTTGCT-GTA3'. Each PCR reaction was conducted in a 20 μL volume which contained 1 μL of first strand cDNA, 2.5 mmol/L MgCl_2 , 200 $\mu\text{mol/L}$ of each dNTP, 0.04 u/ μL Taq DNA polymerase, and 1.0 μM primers. The conditions for DNA amplification consisted of an initial denaturation step of 5 min at 95°C , followed by 50 cycles of 1 min at 95°C , 30 s at 59°C , 1 min at 72°C , and finally, 7 min at 72°C . Half of the PCR samples were separated by electrophoresis on a 2% agarose gel, which was then stained with ethidium bromide (0.5 $\mu\text{g/mL}$). The nucleotide sequences of the amplified products were

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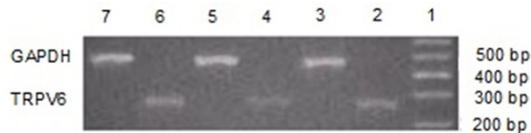


Figure 1. Expression of calcium channel TRPV6 mRNA in the placentas of women with early-onset PE, late-onset PE, and normal women. Lane 1: markers; lane 2: TRPV6 mRNA in a normal placenta; lane 3: GAPDH in an abnormal placenta; lane 4: TRPV6 in early-onset PE; lane 5: GAPDH in early-onset PE; lane 6: TRPV6 in late-onset PE; lane 7: GAPDH in late-onset PE.

analyzed using an ABI310 autosequencer (Applied Biosystems; Foster City, CA, USA).

Immunohistochemistry

The samples of placental tissue were thawed in TRIzol reagent (Thermo Fisher Scientific; Waltham, MA, USA) and minced with razor blades to disperse the tissue fragments. Following an antigen retrieval process, the tissue sections were incubated with polyclonal rabbit anti-TRPV6 polyclonal antibody, and antigen-bound primary antibody was detected using a standard avidin-biotin immunoperoxidase complex. Samples of normal intestinal tissue served as positive control samples. Negative control samples consisted of tissue that had not been treated with the primary antibody.

Immunohistochemical scoring

Tissue microarray slides were concurrently evaluated by two independent pathologists who were blinded to the patients' characteristics. Disagreements between the pathologists were resolved by a third independent observer who determined the final result. The degree of TRPV6 immunoreactivity was graded based on the staining intensities of trophoblastic cell membranes and cytoplasmic compartments. The staining index (range = 0-7) was calculated by adding the scores for the intensity of TRPV6-positive staining (negative, 0; weak, 1-2; moderate, 3-4; strong, > 4) and the percentage of TRPV6-positive cells (< 5%, 0; 5%-24%, 1; 25%-50%, 2; 51%-74%, 3; \geq 75%, 4). Three microscope fields on each slide were observed and scored; and the final score for each slide was the same as the score given for the majority of the observed fields. Thus, > 2 fields on each slide had to receive the same score if the total slide was to be scored; otherwise, additional

fields on the same slide were selected for further observation until > 2 fields received the same score.

Cell culture

HTR-8/SVneo cells (trophoblastic cells present at an early stage of pregnancy) were cultured in RPMI-1640 medium (Gibco BRL; NY, USA) supplemented with 100 U/mL penicillin and streptomycin (Gibco BRL), 5% fetal bovine serum (FBS; Gibco BRL), and 100 U/mL penicillin/streptomycin (Gibco) at 37°C in an atmosphere of 5% CO₂.

Proliferation assay

Ruthenium red, a TRPV6 channel blocker, was dissolved in DMSO to produce a 100 μ M stock solution that could be diluted to the desired concentration with culture medium immediately before use. A 100 μ L aliquot of suspended cells (1×10^5 cells/mL) was placed into each well of flat-bottomed 96-well culture plates, which were then incubated for 24 h in an atmosphere of 5% CO₂ at 37°C. After incubation, 50 μ L of either ruthenium red at the desired concentration or culture medium was added to each well. A well containing only cells plus medium served as a positive control, and a well which contained 200 μ L of medium alone without cells or reagent served as a negative control. The culture plates were incubated for 24 h, 48 h, and 72 h; after which, the MTT assay was used to analyze the effect of ruthenium red on the proliferation of the HTR-8/SVneo trophoblastic cells. A 20 μ L volume of the MTT dye (5 mg/mL) was added to each well, and four hours later, the medium in each well was discarded, and 150 μ L of DMSO was added. The absorbance (A) of each well at 540 nm was read using an automatic multi-well spectrophotometer (Bio-Rad-Coda; Richmond, CA, USA). The negative control well was used for zeroing absorbance. The percentage of proliferating cells was calculated using the background-corrected absorbance as follows: proliferation inhibition rate = $(1 - A \text{ of experimental well} / A \text{ of positive control well}) \times 100\%$. Each experiment was performed at least three times, and representative data are presented.

Intracellular Ca²⁺ measurements

A Fluo-4 NW Calcium Assay kit (Invitrogen; Carlsbad, CA, USA) was used to measure intra-

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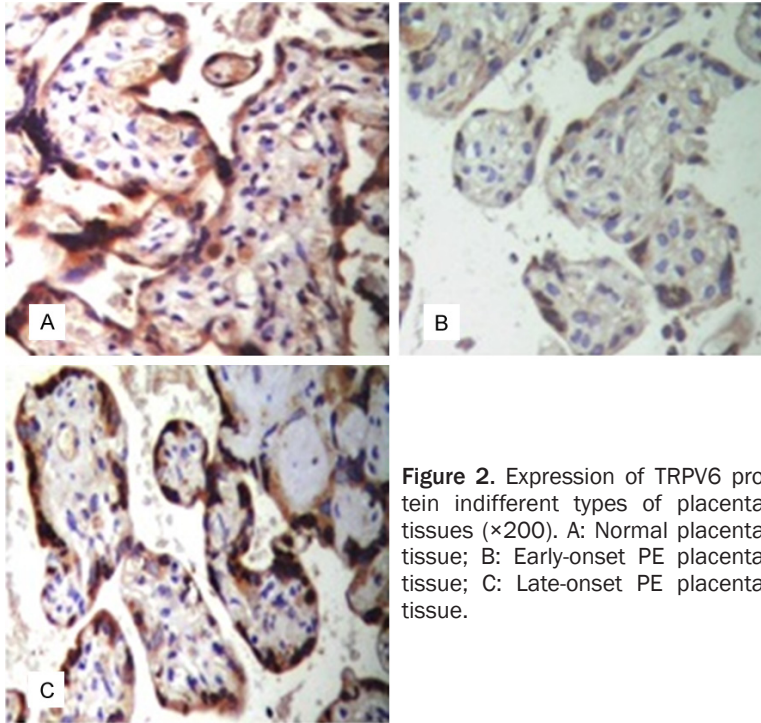


Figure 2. Expression of TRPV6 protein indifferent types of placental tissues ($\times 200$). A: Normal placental tissue; B: Early-onset PE placental tissue; C: Late-onset PE placental tissue.

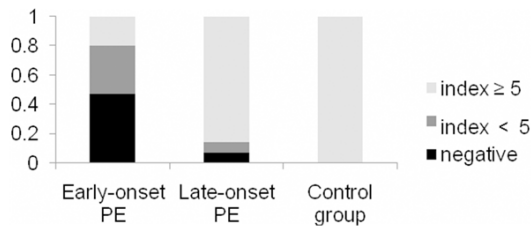


Figure 3. Percentage of TRPV6-positive cells in the 3 types of placental tissue.

cellular Ca^{2+} concentrations on a NOVO star Microplate Reader (BMG Labtech; Offenburg, Germany) according to the manufacturer's protocol. Briefly, $\sim 5 \times 10^4$ cells were cultured overnight in each well of a 96-well plate. On the following day, the growth medium in each well was replaced with 100 μL of Fluo-4 dye solution containing probenecid to prevent extrusion of the dye out of cells. The plate was then incubated for 30 min at 37°C , and then at room temperature for an additional 30 min. The loaded cells were placed in the measurement position in the NOVO Star Microplate reader, and the plate containing ruthenium red was placed in the reagent plate position. The assays were conducted using excitation and emission wave lengths of 494 nm and 516 nm, respectively. Prior to sample injection, three measurements were performed to establish the baseline. The

Ca^{2+} -rich solution contained 140 mM NaCl, 4.7 mM KCl, 1 mM MgCl_2 , 1.3 mM CaCl_2 , and 10 mM HEPES, and had a pH of 7.4. Thapsigargin (Sigma-Aldrich; St. Louis, IL, USA), an intracellular Ca^{2+} pump blocker, was used at a concentration of 100 nM to deplete intracellular Ca^{2+} stores.

Statistical analysis

Data were processed using SPSS19 statistical software and expressed as mean \pm standard derivation (SD). Paired comparisons were performed using variance analysis and tested with the Student's t-test. Values with $P < 0.05$ were considered statistically significant.

Results

TRPV6 gene expression in placental tissue

RT-PCR was used to examine the expression of TRPV6 channel mRNA in human placental tissue. **Figure 1** shows that the PCR amplified a 255-bp TRPV6 channel product and a 452-bp GAPDH product from total placental RNA. Expression of mRNA for TRPV6 was significantly lower in samples of placental tissue obtained from early-onset PE patients (0.45 ± 0.07) when compared with its expression in placental tissue obtained from late-onset PE (0.60 ± 0.06) and normal patients (0.64 ± 0.08). There was no difference between the levels of TRPV6 mRNA expression in the normal group and late-onset PE group. These findings suggest a reduced expression of TRPV6 channels in early-onset PE placental tissues.

Expression of TRPV6 protein in placental tissue

An immunohistochemical analysis showed that TRPV6 staining was remarkably lower in samples of early-onset PE placental tissue when compared with staining in samples of control placental tissue and late-onset PE placental tissue (**Figure 2**). Normal placental tissue had a TRPV6 staining index ≥ 5 ; therefore, staining indexes of 5, 6 or 7 were considered to represent normal levels of TRPV6 expression, where-

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Table 1. Effect of ruthenium red on the absorbance values of cultured HTR-8/SVneo trophoblastic cells

Groups	24 h	48 h	72 h
Negative control	0.00	0.00	0.00
Positive control	4.02 ± 0.21	4.23 ± 0.22	4.36 ± 0.24
Ruthenium red	20.09 ± 0.24*	31.11 ± 0.26*	47.52 ± 0.25*

Data are presented as the mean percentage ± SEM (n = 10-20). *P < 0.01.

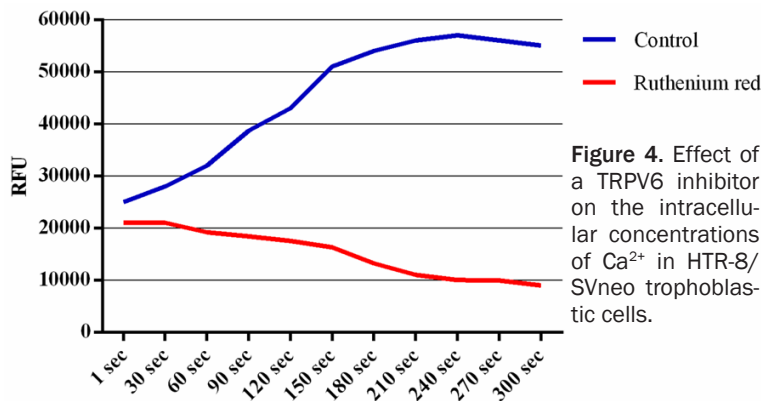


Figure 4. Effect of a TRPV6 inhibitor on the intracellular concentrations of Ca²⁺ in HTR-8/SVneo trophoblastic cells.

as indexes of 0-4 were considered to represent down regulated TRPV6 expression.

When using this index system, down regulated TRPV6 expression was detected in 80% (12 of 15) of placental samples in the early-onset PE group vs. 0% of placental tissues in the control group (P = 0.001), and 13.3% of placental tissues in the late-onset PE group (P = 0.035). There was no significant difference in the level of placental TRPV6 expression in the late-onset PE group when compared with expression in the control group (P = 1.000) (**Figure 3**).

Effect of a TRPV6 channel blocker on trophoblastic cells proliferation

We examined whether a TRPV6 channel blocker (ruthenium red) could influence the proliferation of HTR-8/SVneo trophoblastic cells, and found that it inhibited cell proliferation in a time-dependent manner (**Table 1**). This result verified that TRPV6 channels play an important role in the proliferation of trophoblastic cells.

Effect of ruthenium red on [Ca²⁺] transport

As TRPV6 is a highly Ca²⁺-selective channel protein, inhibition of TRPV6 with ruthenium red might be expected to reduce Ca²⁺ influx. To test this hypothesis, we used Fluo-4 AM to measure

the intracellular Ca²⁺ concentrations in HTR-8/SVneo trophoblastic cells before and after ruthenium red treatment. We found that ruthenium red reduced the mean intracellular Ca²⁺ concentration in HTR-8/SVneo trophoblastic cells by 53 ± 8% from the mean baseline value (**Figure 4**).

Discussion

The TRPV6 gene was first identified in recent genome-wide studies, and since that time, TRPV6 protein has been detected in the normal epithelia of various organs [17, 23]. When compared with its expression in normal tissues and cells, significantly increased levels of TRPV6 mRNA and protein expression have been detected in samples of prostate cancer [11, 12], breast cancer [13-15], and colon cancer tissue [16]. Additionally, aberrant TRPV6 expression has been implicated in the pathogenesis of these cancers. A previous study [24] demonstrated that TRPV6 is expressed in human placental tissue, and is capable of binding Ca²⁺ calmodulin.

Human pregnancy is associated with extensive growth and remodeling of the uterus and placenta. Furthermore, a successful human pregnancy requires cytotrophoblasts from the fetal portion of the placenta to adopt tumor-like properties. Cytotrophoblasts attach the conceptus to the endometrium by invading the uterus, and then initiate blood flow to the placenta by breaching maternal vessels. Therefore, migration and invasion of cytotrophoblasts into the maternal endometrium are key events in human placentation. Inadequate trophoblast invasion is well documented in cases of PE, and is recognized to be the main cause for development of PE.

To investigate TRPV6 involvement in the regulation of trophoblast function and the pathophysiology of PE, we examined the levels of TRPV6 expression in early-onset PE placentas, late-onset PE placentas, and normal placentas. We

found significantly lower levels of TRPV6 expression in the placental tissues of early-onset PE patients, when compared with those levels in late-onset PE patients and control subjects. Interestingly, there was no significant difference between the levels of TRPV6 expression detected in the placentas of late-onset PE patients and the control subjects. It is well-known that early-onset preeclampsia is considered to be a fetal disorder typically associated with placental dysfunction, reduced placental volume, intrauterine growth restriction, abnormal uterine and umbilical artery Doppler evaluation results, low birth weight, multiorgan dysfunction, perinatal death, and adverse maternal and neonatal outcomes [25, 26]. In contrast, late-onset preeclampsia is considered to be a maternal disorder resulting from an underlying maternal constitutional disorder, and is more often associated with a normal placenta, larger placental volume, normal fetal growth, normal uterine and umbilical artery Doppler evaluation, normal birth weight, and more favorable maternal and neonatal outcomes [27-29]. When taken together, such findings suggest that TRPV6 expression in placental tissues might be regulated in cases of early-onset PE.

The early placenta invasion process shares many features with the tumor progression process, as it proceeds through the following steps: epithelia-mesenchyme transition (EMT), tumor growth, and metastasis [30-33]. Indeed, previous studies have demonstrated that TRPV6 is over expressed in a variety of invasive tumor cells, and plays a critical role in tumor cell invasion and metastasis. Another study [12] reported the involvement of TRPV6 channels in LNCaP cell proliferation and their resistance to apoptosis. Inadequate trophoblast invasion has been well documented in cases of PE, and is recognized to be the main cause for development of PE. To investigate TRPV6 involvement in the regulation of trophoblast function and pathophysiology of PE, we assessed the effect of TRPV6 on the proliferation of HTR-8/SVneo cells. We found that a TRPV6 inhibitor (ruthenium red) could reduce the proliferation of HTR-8/SVneo cells, suggesting that TRPV6 plays an important role in promoting the proliferation of trophoblast cells.

Nevertheless, the molecular mechanism by which TRPV6 helps to regulate cell proliferation

remains poorly understood, and requires further investigation. Ca^{2+} is a universal intracellular second messenger involved in numerous biological processes such as signal transduction, neurotransmission, enzyme and hormone secretion, cell cycle regulation, and programmed cell death. Several studies [12, 34] have reported that Ca^{2+} influx through TRPV6 channels modulates the proliferation of transformed cells and alters their resistance to apoptotic death. The transepithelial transport of Ca^{2+} through the syncytiotrophoblast is a passive/active process requiring the participation of various proteins, and previous studies have proven that TRPV6 modulates this transport process [21]. Ca^{2+} levels in fetal blood are significantly lower in TRPV6 knockout mice when compared with their levels in normal mice. This suggests that TRPV6 is crucial for normal fetal development and the prenatal programming of future diseases. In this study, we found that HTR-8/SVneo trophoblastic cells treated with ruthenium red to block TRPV6 had a significantly decreased ability to transport Ca^{2+} . These results indicate that TRPV6 channels play an important role in placental Ca^{2+} transfer, and suggest the involvement of TRPV6 in trophoblastic cell proliferation.

In summary, for the first time, we showed that TRPV6 expression at the mRNA and protein levels was down-regulated in the placentas of early-onset PE patients. We also demonstrated that inhibition of TRPV6 activity by ruthenium red could suppress the proliferation of trophoblast cells. These results suggest that TRPV6 plays an important role in the growth of trophoblast cells, and that down-regulated TRPV6 expression may be involved in the pathogenesis of early-onset PE.

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Disclosure of conflict of interest

None.

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