Original Paper

Activation of PAR-1/NADPH Oxidase/ROS Signaling Pathways is Crucial for the Thrombin-Induced sFlt-1 Production in Extravillous Trophoblasts: Possible Involvement in the Pathogenesis of Preeclampsia

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Key Words

Thrombin • Trophoblast • sFlt-1

Abstract

Backgrounds/Aims: Preeclampsia was characterized by excessive thrombin generation in placentas and previous researches showed that thrombin could enhance soluble Fms-like tyrosine kinase 1 (sFlt-1) expression in first trimester trophoblasts. However, the detailed mechanism for the sFlt-1 over-production induced by thrombin was largely unknown. The purpose of this study was to explore the possible signaling pathway of thrombin-induced sFlt-1 production in extravillous trophoblasts (EVT). **Methods:** An EVT cell line (HRT-8/SVneo) was treated with various concentrations of thrombin. The mRNA expression and protein secretion of sFlt-1 in EVT were detected with real-time polymerase chain reaction and ELISA, respectively. The levels of intracellular reactive oxygen species (ROS) production were determined by DCFH-DA. **Results:** Exposure of EVT to thrombin induced increased intracellular ROS generation and overexpression of sFlt-1 at both mRNA and protein levels in a dose dependent manner. Short interfering RNA (siRNA) directed against PAR-1 or apocynin (an inhibitor of NADPH oxidase) could decrease the intracellular ROS generation and subsequently suppressed the production of sFlt-1 at mRNA and protein levels. **Conclusions:** Our results suggested that thrombin increased sFlt-1 production in EVT via the PAR-1 /NADPH oxidase /ROS signaling pathway. This also highlights the PAR-1 / NADPH oxidase / ROS pathway might be a potential therapeutic target for the prevention of preeclampsia in the future.

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Introduction

Preeclampsia is a complex multi-system obstetric syndrome affecting about 5–10% of pregnant women and remains a leading cause of maternal and perinatal mortality and morbidity. It is characterized by hypertension and significant proteinuria at or after 20 weeks of pregnancy [1].

Dysregulations of angiogenic pathways have been showed to contribute to the inadequate blood supply of the placenta in patients with preeclampsia [2, 3]. Soluble Fms-like tyrosine kinase 1 (sFlt-1) is a splice variant of the vascular endothelial growth factor (VEGF) receptor Flt-1 that lacks the transmembrane and cytoplasmic domains [4]. Previous studies demonstrated that exogenous sFlt-1 administered to pregnant rats could induce a preeclampsia-like phenotype including hypertension, proteinuria, and glomerular endotheliosis in these animals [5, 6]. Moreover, circulating levels of sFlt-1 were elevated in the serum of women with preeclampsia [7–9], and this elevation preceded the development of clinical signs by 4–6 weeks. A recent pilot study performed by Thadhani et al. [10] found that lowering circulating sFlt-1 by using extracorporeal apheresis was able to extend 3 preeclamptic pregnancies by 2–4 weeks, all of which resulted in healthy deliveries with no neonatal or maternal morbidity. Taken together, these observations suggested that excess sFlt-1 production might be associated with the development of preeclampsia.

Thrombin is a multifunctional serine protease generated at the site of vascular injury that transforms fibrinogen into fibrin, activates blood platelets and elicits multiple effects on a variety of cell types including extravillous trophoblast cells [11], endothelial cells [12], vascular smooth muscle cells [13], and monocytes [14]. It is widely known that uteroplacental hemorrhage [15], fibrin deposition [16], and infarction [17] are commonly observed in established preeclampsia. Moreover, uterine bleeding or the presence of hematoma at implantation sites in the first trimester is known to be associated with the later development of preeclampsia [18]. These conditions would generate excess thrombin. A recent research demonstrated that excess placental thrombin formation may be involved in the pathogenesis of preeclampsia [19]. A growing body of evidence demonstrated that plasma thrombin exerted its wide-range effect through activating specific proteinase-activated receptors (PAR, eg: PAR1, PAR3 and PAR4), NADPH oxidase system and inducing intracellular ROS production in various cell types [20–23]. For instance, prior studies suggested that endothelial dysfunction induced by excess formation of thrombin was associated with NADPH oxidase-dependent ROS generation. Inhibiting thrombin-PAR1 interaction or blocking NADPH oxidase activity could reduce the intracellular ROS generation and therefore improved endothelial function [12, 20].

Placenta was thought to be the primary source of circulating sFlt-1 in women with preeclampsia. A previous study suggested that thrombin could enhance sFlt-1 expression in first trimester trophoblasts [11], establishing a possible link between thrombin and preeclampsia. However, the detailed mechanism for the sFlt-1 over-production caused by thrombin in placenta was largely unknown. The aim of the present study was to determine the possible role of the PAR-1/ NADPH oxidase signaling pathways of thrombin mediated sFlt-1 expression by trophoblasts.

Materials and Methods

The Institutional Review Board at the Nanfang Hospital, Southern Medical University approved the study protocol and all procedures.

Cell culture

First trimester EVT cell line (HTR-8/SVneo cells) was a kind gift from Dr Charles H. Graham (Queen's University, Ontario, Canada). The cells were cultured in RPMI-1640 (HyClone, South Logan, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin

(HyClone), at 37 °C plus continuous supplement of 5% CO₂. Before experiments, HTR-8/SVneo cells were cultured in RPMI-1640 supplemented with 2.5% FBS for 24 h.

Intervention with Cell Cultures

EVT were rinsed with phosphate-buffered saline solution, replenished with fresh serum-free media, and cultured for an additional 24 hours. To evaluate the effect of thrombin on sFlt-1 production in EVT, the cells were incubated with serum-free medium and different concentrations of thrombin (0.1, 1, and 10 U/ml; Sigma-Aldrich). We measured the intracellular ROS production of EVT treated with various concentrations of thrombin (0.1, 1, and 10 U/ml) in indicated time (12 hours) or thrombin (10 U/ml) over a time course (3, 6, 12 and 24 hours). In addition, we observed the effect of specific siRNA (inhibit different PAR receptors expression) or various potential enzymes [24] including inhibitors of NADPH oxidase (apocynin, 500 µg/ml), nitric oxidase synthase (L-NNMA, 1000 µmol/l), xanthine oxidase (allopurinol, 100 µmol/l) and mitochondrial respiratory chain complexes (rotenone, 250 µmol/l), on EVT in vitro and to explore whether specific siRNA or these enzymes may regulate the effect of thrombin on the expression of sFlt-1 in EVT.

siRNA transfection

We used validated siRNA directed against PAR-1 (Cat. # 4390771), PAR-3 (Cat. # AM51331) and PAR-4 (Cat. # AM16708) mRNA from Ambion. Briefly, when EVT cells reached 70%-85% confluence, they were trypsinized and centrifuged at 90×g at 4°C for 10 min. 1×10⁶ cells/condition were resuspended in 100 µL of Nucleofector Solution (Amaxa/Lonza, Allendale, NJ) with 100 nmol/L siRNA. Electroporation transfection process was performed using the appropriate cell type program X005 in Nucleofector II (Amaxa Biosystems, Germany), as described previously [25]. After that, we immediately add 500 µl of pre-warmed RPMI-1640 with 10% FBS into the transfected cells, which were seeded into 6-well plates for 48 hours and then prepared for further analysis. Successful silencing of PAR-1, PAR-3, and PAR-4 were confirmed by Western blotting (Fig 1).

RNA extraction, reverse transcription, and real-time quantitative polymerase chain Reaction

Total RNA was extracted from EVT with the use of an RNeasy mini kit (Qiagen). One µg total RNA was reverse transcribed in a 20 µL volume with the use of ReverTraAcea (Toyobo) according to the manufacturer's instructions. Real-time quantitative polymerase chain reaction (PCR) and data analysis were performed using Lightcycler (Roche Diagnostic) according to the manufacturer's instructions. Two µL cDNA in 20 µL volume was amplified with the use of oligonucleotide primers based on human sFlt-1 sequence. sFlt-1 Primers (sense 5'-GCA CCT TGG TGG CTG ACT-3', antisense 5'-GGG CCC GGG GGT CTC ATT ATT-3') were used to amplify a 643-bp product. The PCR conditions for the mRNA were as follows: 45 cycles at 95°C for 10 seconds, 63°C for 10 seconds, and 72°C for 27 seconds. All PCRs were followed with melting curve analysis. Relative gene expression was measured using [$\Delta\Delta C(t)$] method and normalized to the geometric mean of two housekeeping genes: glyceraldehyde dehydrogenase (*GAPDH*) (sense 5'-AGA TCA TCA GCA ATG CCT CC -3', antisense 5'-CAT GAG TCC TCC CAC GAT AC -3') and TATA-binding protein (*TBP*) (sense 5'-CAC GAA CCA CGG CAC TGA TT -3', antisense 5'-TTT TCT TGC TGC CAG TCT GGA C -3').

Measurement of protein secretions in culture supernatants

Conditioned culture media were centrifuged and stored at -80°C until assay. Concentrations of sFlt-1 in supernatants were measured with the use of its specific ELISA kits (Quantikine; R&D Systems) according to the manufacturer's instructions.

Measurement of intracellular ROS production

The levels of intracellular ROS production were determined by measuring the fluorescence of 5 (and 6)-chloromethyl-2',7'-dichlorodifluorescein diacetate (DCF, Molecular Probe, Carlsbad, CA). Briefly, EVT were pre-incubated for 30 min with 1 nmol/L DCF in PBS without Ca²⁺ and Mg²⁺. The cells were then incubated with various concentrations of thrombin (0.1, 1, and 10 U/ml) [11] in indicated time (12 hours) or thrombin (10 U/ml) over a time course (3, 6, 12 and 24 hours) [24]. One milliliter aliquots of the cells were removed for fluorescence intensity analysis on a flow cytometry (BD FACSCalibur system, Franklin Lakes, NJ). To verify the sources of ROS generation, EVT were pre-incubated for 10 min with apocynin, L-NAME, allopurinol or rotenone as mentioned above (all from Sigma).

Fig. 1. HTR-8/SVneo cells were transfected with PAR-1, PAR-3, PAR-4 siRNA or scramble siRNA (siNC). Western blot was performed to analyze the expression of endogenous PAR-1, PAR-3 and PAR-4 in HTR-8/SVneo cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to verify equivalent loading. (* compared with control group, $P < 0.05$; # compared with control group, $P < 0.01$).

Fig. 2. (A) Effects of thrombin on sFlt-1 mRNA expression. (B) Effects of thrombin on sFlt-1 secretion. Error bars depict SD. (* compared with control group, $P < 0.05$; ** compared with control group, $P < 0.01$).

Statistical Analysis

All experiments were performed 3 times or more independently and all the values were expressed as the mean $\pm$ standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA) with the application of the Dunnett's test. Differences were considered as statistically significant at $P < 0.05$.

Results

Effect of thrombin on sFlt-1 mRNA expression and protein secretion in EVT

As shown in Figure 2A&B, exposure of EVT to thrombin significantly induced higher expressions of sFlt-1 at both the mRNA level and protein level in a dose dependent manner.

Thrombin increased intracellular ROS generation in EVT through PAR-1 / NADPH dependent pathway

Thrombin increased the intracellular ROS production in a time- and dose-dependent manner when compared with cells cultured in medium alone (Fig. 3A&B). Furthermore, thrombin induced intracellular ROS production could be blocked by pretreating EVT with inhibitor of NADPH oxidase (apocynin), but not by the inhibitors of nitric oxidase synthase (L-NNMA), xanthine oxidase (allopurinol) or the inhibitor of mitochondrial respiratory chain complexes (rotenone), suggesting that thrombin induced intracellular ROS production in EVT was dependent on NADPH oxidase activation (Fig. 3C). Moreover, specific siRNA was used to block the PARs expression in EVT. As shown in Fig. 3C, it was PAR-1 specific siRNA but not

Fig. 3. Thrombin increased PAR-1 / NADPH-dependent intracellular ROS production in HTR-8/SVneo cells. Intracellular ROS production was assessed using DCFH-DA. (A) HTR-8/SVneo cells were treated with thrombin (10 U/ml) for 3, 6, 12 and 24 hours. (B) HTR-8/SVneo cells were treated with various concentration of thrombin (0.1, 1.0, 10 U/ml) for 12 hours. (C) HTR-8/SVneo cells were transfected with PAR receptor or scramble siRNA (siNC) for 24 h or pretreated with apocynin (NADPH oxidase inhibitor, 500µg/ml), L-NNMA (nitric oxidase synthase 1000µmol/l), allopurinol (xanthine oxidase inhibitor, 100µmol/l), rotenone (inhibitor of mitochondrial electron transport complex I, 250µmol/l) for 10 minutes followed by incubation with thrombin (10 U/ml) for 12 hours. Data are representative of three independent experiments. (* compared with control group, $P < 0.05$; ** compared with control group, $P < 0.01$; # compared with thrombin (10 U/ml) group, $P < 0.05$; ## compared with thrombin (10 U/ml) group, $P < 0.01$).

other PARs siRNA or the scramble siRNA significantly blocked the enhanced ROS generation by thrombin, demonstrating that thrombin could bind PAR-1 to induce intracellular ROS generation in EVT.

Thrombin-induced sFlt-1 production in EVT was dependent on intracellular ROS generation via activation of PAR-1 and NADPH oxidase

We then examined whether intracellular ROS generation was necessary for thrombin-induced sFlt-1 production in EVT. As demonstrated in Figure 4, both sFlt-1 mRNA expression and protein secretion in EVT treated with thrombin could be inhibited by apocynin in a dose-dependent manner, but not by L-NNMA, allopurinol, rotenone. These data suggested that intracellular ROS generation through NADPH oxidase may be required for thrombin-induced sFlt-1 production in EVT. In addition, thrombin induced sFlt-1 production in EVT could be also blocked by PAR-1 specific siRNA, but not other PARs siRNA or the scramble siRNA, indicating that thrombin induced sFlt-1 production was mainly mediated by a PAR-1 dependent intracellular ROS generation pathway.

Fig. 4. (A) Effects of various inhibitors or siRNAs on thrombin(10 U/ml) induced sFlt-1 mRNA expression. (B) Effects of various inhibitors or siRNAs on thrombin(10 U/ml)induced sFlt-1 secretion. TH: thrombin (10 U/ml); apocynin (100; 250; 500): (NADPH oxidase inhibitor, 100 µg/ml; 250 µg/ml; 500 µg/ml), L-NNMA: (nitric oxide synthase 1000µmol/l), allopurinol: (xanthine oxidase inhibitor, 100µmol/l), rotenone: (inhibitor of mitochondrial electron transport complex I, 250µmol/l). Error bars depict SEM. (# compared with control group, $P < 0.05$; ## compared with control group, $P < 0.01$).

Discussion

Although abnormal sFlt-1 production is considered to be one of the crucial factors in the development of preeclampsia, the molecular signaling pathways that regulate the production of sFlt-1 during preeclampsia are still not completely understood. In the present study, we demonstrated that thrombin increased the expression of sFlt-1 in trophoblasts by activation of PAR-1/NADPH oxidase/ROS signaling pathways and might thereby be involved in the development of preeclampsia.

Previous study showed that thrombin could activate intracellular signaling pathways by interacting with transmembrane domain G-protein-coupled receptors (Protease activated receptors, PARs). Four members of PARs have been cloned and designated PAR-1, PAR-2, PAR-3, and PAR-4 [26]. Three of these members, PAR-1, PAR-3, and PAR-4 were cleaved by thrombin, whereas PAR-2 was cleaved by trypsin. So one fundamental issue to be addressed was exactly how thrombin transmit their signals across the plasma membrane to elicit cellular activities. Erez et al. [27] showed that significantly stronger PAR-1 expression in the placenta of preeclampsia than their normal controls and suggested PAR-1 as a crucial mediator of thrombin induced coagulation and inflammation in preeclampsia. In this study, we provided evidence demonstrating that PAR-1 but not other PARs was required for thrombin-induced sFlt-1 production in EVT, since we could not inhibit thrombin-induced sFlt up-regulation by PAR3 and PAR4 receptor specific siRNA. These data suggested that PAR1 was involved in thrombin-induced sFlt expression and release from EVT.

In addition, a growing body of studies indicated that thrombin elicited its wide-range cellular effect through activating the PAR-1/NADPH oxidase system and inducing intracellular ROS in several different cell types [11-14, 20]. Mounting evidence suggested that PAR-1 participated actively in various vascular and inflammatory diseases [28, 29]. A recent study observed that protection effect against thrombin-induced endothelial dysfunction through inhibiting thrombin-PAR1 interaction [30].

Cariello et al. [23] showed that excess formation of thrombin was associated with NADPH oxidase-dependent ROS generation in hemodialysis patients and suggested that

inhibition of NADPH oxidase may prevent thrombin-mediated damage in these patients. Here, we also observed that thrombin increased the intracellular ROS production in a time- and dose-dependent manner in EVT. Moreover, we demonstrated that apocynin (inhibitor of NADPH oxidase) but not other oxidase inhibitor, could decrease the intracellular ROS production and eventually inhibited thrombin-induced sFlt-1 expression, which suggested intracellular ROS generation by NADPH oxidase activation might be responsible for the thrombin-induced sFlt-1 expression.

Prior studies found that hypoxia and insufficient placentation, speculated to occur in the first trimester, could increase trophoblast sFlt-1 production, and caused consequently systemic and local endothelial dysfunction, which might induce further placental sFlt-1 production and enhanced placental hypoxia/ischemia, contributing to the 'vicious' cycle [5, 31]. Accumulating evidence also demonstrated that hypoxic conditions in the placenta could promote oxidative stress [5, 31, 32]. Moreover, several recent researches have observed that oxidative stress could accelerate thrombin formation and enhance vascular reactivity to thrombin [33, 34]. Thereby, excessive thrombin formation in the placenta might enhance sFlt-1 expression by trophoblasts and could be involved in this 'vicious' cycle.

One of the limitations in the present study was we only focused on the possible signaling pathways in EVT, a major cellular source of sFlt-1 in implantation sites. Prior studies have demonstrated that first trimester deciduae cells and peripheral blood monocytes could also express sFlt-1 [14, 20]. Thereafter, investigations were warranted to explore the possible role of thrombin on the sFlt-1 expression in the decidua cells and peripheral blood monocytes in the near future.

In summary, the present study demonstrated that excess formation of thrombin might contribute to the pathogenesis of preeclampsia by promoting sFlt-1 production in EVT, probably through activation of PAR-1/NADPH oxidase/ROS dependent pathway. We proposed understanding the possible mechanism of thrombin on EVT might be an important step toward development of new strategies and interventions for trophoblasts biological dysfunctions and preeclampsia. This also highlights the PAR-1 / NADPH oxidase / ROS pathway may be a potential therapeutic target for the prevention of preeclampsia in the future.

Disclosure Statement

The authors have no competing interests to declare.

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