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PAR-2 triggers placenta-derived protease-induced altered VE-cadherin reorganization at endothelial junctions in preeclampsia

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ABSTRACT

PAR-2 is a G-protein coupled protease receptor whose activation in endothelial cells (ECs) is associated with increased solute permeability. VE-cadherin is an endothelial-specific junction protein, which exhibits a disorganized distribution at cell junction during inflammation and is a useful indicator of endothelial barrier dysfunction. In the present study, we tested the hypothesis that PAR-2 activation mediates placenta-derived chymotrypsin-like protease (CLP)-induced endothelial junction disturbance and permeability in preeclampsia (PE). PAR-2 and VE-cadherin were examined by immunofluorescent staining. Specific CLP induced PAR-2 activation and altered VE-cadherin distribution was assessed following depletion of protease chymotrypsin in the placental conditioned medium and after PAR-2 siRNA. VE-cadherin assembly was determined by treating cells with protease chymotrypsin and/or the specific PAR-2 agonist SLIGKV-NH2. Our results showed: 1) placental conditioned medium not only disturbed VE-cadherin distribution at cell junctions but also activated PAR-2 in ECs; 2) PAR-2 siRNA blocked the placental conditioned medium induced PAR-2 upregulation and disorganization of VE-cadherin at cell junctions; 3) PAR-2 agonist induced PAR-2 activation and VE-cadherin reorganization were dose-dependent; and 4) PAR-2 agonist could stimulate ERK1/2 activation. These results strongly suggest that proteases produced by the placenta elicit endothelial barrier dysfunction via a PAR-2 signaling regulatory mechanism in PE.

1. Introduction

Increased endothelial permeability is a hallmark of endothelial dysfunction in preeclampsia, a hypertensive and multi-system disorder in human pregnancy. In the systemic microvasculature, increased endothelial permeability leads to interstitial edema. By studying the mechanisms underlying endothelial barrier dysfunction in preeclampsia, we found that increased endothelial permeability is associated with decreased expression of endothelial-specific junction protein VE-cadherin and an altered distribution of VE-cadherin at endothelial junctions [1]. We have now identified that chymotrypsin-like proteases (CLP) derived from the placenta promote endothelial inflammatory responses [2,3], disintegrate VE-cadherin binding between neighbor endothelial cells [4], and diminish endothelial barrier function [5]. However, the cellular response and the mechanism of placenta-derived CLP induced endothelial barrier dysfunction in preeclampsia remain largely unknown.

Protease-activated receptors (PARs) are members of the G-protein-coupled receptor super-family that are activated by the proteolytic cleavage within the N terminal domain. PARs exist in 4 isoforms. PAR-1, -3, and -4 are cleaved and activated by thrombin while PAR-2 is cleaved and activated by trypsin, chymotrypsin, and other CLP's including chymase. PARs are now emerging as important modulators of diverse biological functions in the vascular system such as endothelial-dependent vessel contraction [6]. PAR-2 activation has been reported in various cell types including endothelial cells, platelets, leukocytes, and fibroblasts and PAR-2 activation modulates numerous physiological and pathophysiological conditions [7,8]. In endothelial cells, PAR-2 activation induces tissue factor expression and von Willebrand factor release from Weibel-Palade bodies [9]. PAR-2 activation also promotes blood coagulation [10], leukocyte recruitment, and vascular permeability [11]. Thus, PAR-2 activation represents an important hallmark of inflammatory response in vascular endothelial cells.

We previously reported that soluble factors derived from the preeclamptic placenta could promote PAR-2 activation in

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endothelial cells [8], and that placenta-derived CLP could disturb endothelial adhesion protein VE-cadherin spatial distribution to increase endothelial permeability [4]. However, it is not known if PAR-2 activation is involved in altered VE-cadherin distribution induced by CLP-derived from preeclamptic placenta. The objective of this study intended to establish the link of placental-derived CLP – endothelial dysfunction and increased endothelial permeability in preeclampsia. In the present study, we employed an *in vitro* cell culture model to specifically investigate and test the hypothesis that altered VE-cadherin expression and distribution induced by placenta-derived CLP is mediated through PAR-2 activation in endothelial cells and to explore the potential signaling cascade event that is involved in endothelial barrier dysfunction in preeclampsia.

2. Materials and methods

2.1. Chemicals and reagents

Endothelial cell growth medium (EGM) was purchased from Lonza Walkersville, Inc. (Walkersville, MD). PAR-2 agonist SLIGKV-NH2 was purchased from Bachem (Buberdorf, Switzerland). Antibodies for PAR-2, VE-cadherin, ERK and pERK were purchased from Santa Cruz (San Diego, CA). β-actin antibody was from Sigma Chemicals (St. Louis, MO) and chymotrypsin antibody was from Abcam (Cambridge, MA). Cy3 labeled donkey anti-mouse IgG (H + L) was from Jackson Immunoresearch laboratories Inc. (Westgrove, PA). PAR-2 siRNA (sc-36188) and scrambled siRNA were purchased from Santa Cruz. Dulbecco's Modified Eagle's Medium (DMEM), horseradish peroxidase (HRP), guaiacol, hydrogen peroxide (H_2O_2), and protease inhibitors were from Sigma. All other reagents were obtained from Sigma unless otherwise noted.

2.2. Tissue collections

Placentas from normal and preeclamptic pregnant women were obtained at the main hospital, Louisiana State University Health Sciences Center – Shreveport (LSUHSC-S), LA. Normal pregnancy was defined as a pregnancy with normal blood pressure (<140/90 mmHg), negative proteinuria, and absence of obstetrical and medical complications. Preeclampsia was defined as follows: sustained systolic blood pressure of ≥140 mmHg or a sustained diastolic blood pressure of ≥90 mmHg on two separate readings; proteinuria measurement of 1+ or more on dipstick, or 24 h urine protein with ≥300 mg in the specimen. None of the patients had signs of infection and smokers were excluded. Tissue collections were approved by the Institutional Review Board (IRB) for Human Research at LSUHSC-S. Umbilical cords from normal placentas were used to isolate HUVECs. Placentas from preeclamptic pregnancies were used to prepare placental conditioned medium.

2.3. Endothelial cell isolation and culture

HUVECs were isolated by collagenase digestion of umbilical cord vein of placentas delivered by normal term pregnant women as previously described [12]. Isolated HUVECs were cultured with EGM containing recombinant human epithelial growth factor (rhEGF), hydrocortisone, gentamicin sulfate/amphotercin-B, bovine brain extract, and 2% fetal bovine serum (FBS). Cells used for fluorescent staining were grown on glass coverslips in 24 well/plates. Cells used for protein expression by Western blot were grown in 6 well/plates or T25 flasks. First passage cells were used in all experiments.

2.4. Placental conditioned medium preparation

Placental conditioned medium was prepared by culturing villous tissue from preeclamptic placentas as previously described [2]. Briefly, Placental tissue was gently separated by sterile dissection from different cotyledons, excluding chorionic and basal plates, and washed repeatedly with phosphate buffered saline (PBS) to remove blood. Villous tissue explants 500mg/well in 6 well/plates were incubated with 7 ml DMEM containing penicillin, streptomycin, and amphotericin B without serum. The incubation was carried out for 48 h at 37C in an incubator gassed with 95% air-5% CO_2 (Forma Scientific, Inc., Marietta, OH). Medium samples were collected at the end of incubation as conditioned medium (CM) and stored at −80°C freezer. In general, conditioned medium was used within 6 months after preparation. Pooled conditioned medium from 2 to 3 placental cultures were used to treat endothelial cells in each treatment assay and conditioned medium from at least 15 placentas were used in this study.

2.5. PAR-2 siRNA transfection

Transfections were conducted using siPORT™ Lipid Transfection Agent (Ambion Inc. Austin, TX) according to the manufacturer's instructions. Briefly, 0.5 nmol of siRNA was diluted with Opti-MEM I medium without serum and antibiotics and mixed with siPORT™ Lipid Transfection Agent. Cells were transfected at 37C gassed with 95% air-5% CO_2 for 4 h and then medium was replaced with fresh EGM containing 2% FBS. Scrambled siRNA was transfected as control. Cells were treated with conditioned medium 48 h after transfection.

2.6. Immunofluorescent staining

Endothelial PAR-2 and VE-cadherin distribution and abundance were determined by immunofluorescent staining. Briefly, confluent endothelial cells grown on glass coverslips were treated with placental conditioned medium or PAR-2 agonist. After treatment, cells were fixed with 95% ethanol, permeabilized with 50% acetone, and then stained with monoclonal antibodies against PAR-2 or VE-cadherin. Cy3 labeled donkey anti-mouse IgG (H + L) was used as the secondary antibody. Cells stained without primary antibody served as negative control. Stained cells were examined by fluorescent microscopy (Olympus IX71, Tokyo, Japan). Images were recorded using a digital camera with Picture Frame computer software (Optronics Inc., Sunnyvale, CA).

2.7. Protein expression

Endothelial VE-cadherin, ERK, and pERK abundance were examined by Western blotting. An aliquot of total cellular protein (10 μg of each sample) was subjected to electrophoresis (Bio-Rad, Hercules, CA) and then transferred to Hybond-protein transfer membrane (Amersham Corp, Arlington Heights, IL). The membrane was blocked with 5% milk in phosphate buffered saline and then probed with primary antibody at 4 °C overnight. Proteins were visualized using enhanced chemiluminescent (ECL) detection Kit (Amersham Corp). The membrane was stripped before being probed with β-actin antibody (used as the loading control for each sample).

2.8. Endothelial permeability assay

Endothelial permeability was determined by measuring the passage of horseradish peroxidase (HRP) across confluent endothelial monolayers grown on polycarbonate cell culture inserts as we previously described [1,13]. The HRP enzymatic activity was measured by a spectrophotometer with the wavelength at 470 nm (Ultraspec 3000, Pharmacia Biotech). Data were calculated as OD470 nm sample-OD470 nm blank, and expressed as OD470 nm for permeation of HRP across transwell filters.

2.9. Statistical analysis

Data are expressed as mean ± SE and analyzed by analysis of variances (ANOVA) using StatView software (Cary, NC). Student-Newman-Keuls test was used as a post hoc test. A probability level of less than 0.05 was considered statistically significant.

3. Results

3.1. PAR-2 activation is associated with disruption of VE-cadherin expression at endothelial junction

Our previous study showed that disturbed VE-cadherin distribution at cell junction was detected after 2hr treatment by preeclamptic placental conditioned medium and internalization of VE-cadherin was detected after 24hr treatment [14]. PAR-2 is a G-protein coupled receptor. The binding of ligands and G-proteins to receptors is an early event of cell response. To focus on the changes of VE-cadherin distribution at cell junctions, we first determined if PAR-2 activation modulates VE-cadherin expression and distribution in endothelial cells. Confluent endothelial cells were treated with preeclamptic placental conditioned medium for 30 min, 1, and 2 h and endothelial expression of PAR-2 and VE-cadherin were determined by immunofluorescent staining. Representative images for PAR-2 and VE-cadherin expression and distribution at 2 h treatment are shown in Fig. 1A. PAR-2 expression was detected in cells treated with preeclamptic placental conditioned medium, but not in control cells. VE-cadherin expression was detected at junctional regions