



Technical note

Elevated protease HtrA4 in the maternal circulation of preeclampsia may contribute to endothelial barrier disruption by cleaving key junctional protein VE-cadherin



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ABSTRACT

Preeclampsia is hallmarked by systemic endothelial dysfunction, including increased endothelial permeability and oedema. Placenta-derived factors in maternal blood contribute to endothelial barrier impairment, but molecular mechanisms are unclear. HtrA4 is a placenta-specific protease that is secreted into the maternal circulation and elevated in early-onset preeclampsia. In this study, we found HtrA4 cleaved the key endothelial junctional protein VE-cadherin *in vitro*. HtrA4 at concentrations found in preeclampsia also cleaved VE-cadherin in HUVECs as an endothelial model, disrupted cell-cell connections and induced intercellular gaps. These results provide critical insights into understanding the molecular mechanisms of endothelial barrier disruption in preeclampsia.

1. Introduction

Preeclampsia (PE) is a serious pregnancy complication characterised by new-onset hypertension, with proteinuria and/or maternal organ dysfunction after 20 weeks of gestation [1]. PE is hallmarked by systemic endothelial dysfunction [2], including endothelial inflammation, vasoconstriction and oedema [3–5]. The endothelium forms a barrier between the bloodstream and underlying tissues, and its integrity regulates vascular permeability. Abnormal levels of placental factors circulating in preeclamptic blood disturbs endothelial integrity and increases vascular permeability [5,6], however, causal factors and mechanisms are yet to be identified.

VE-cadherin is a key adherens junctional protein that is specifically expressed in endothelial cells [7]. It plays a critical role in controlling endothelial cell-cell contacts and vascular permeability, and disruptions to VE-cadherin will disturb the endothelial barrier function [8]. VE-cadherin is also disrupted in pre-eclamptic women. In human umbilical vascular endothelial cells (HUVECs) isolated from preeclamptic patients, VE-cadherin protein is greatly reduced, cellular morphology altered and endothelial permeability increased [5,9]. It is further suggested that VE-cadherin may be disrupted post-transcriptionally by placenta-derived chymotrypsin-like proteases [9,10].

We have previously reported that HtrA4, a chymotrypsin-like serine protease, is expressed specifically by the human placenta, secreted into the maternal circulation, and significantly elevated in early-onset PE [11]. We have also demonstrated that HtrA4 increases endothelial permeability *in vitro* [11]. The present study aimed to determine if HtrA4, at concentrations found in the preeclamptic circulation, could cleave VE-cadherin in endothelial cells and disrupt cell-cell contacts.

2. Materials and methods

2.1. *In vitro* cleavage of recombinant VE-cadherin by HtrA4

Recombinant human VE-cadherin was obtained from R&D Systems (Minneapolis, Minnesota, USA) and recombinant human HtrA4 was purified from Expi293F cells following transfection. VE-cadherin (100 ng) was incubated with HtrA4 (1 ng) in a cleavage buffer [50 mM Tris-HCl (pH8.0), 200 mM NaCl, and 0.25% (w/v) CHAPs] at 37 °C for 30 min, 2 and 6 h respectively. Controls included VE-cadherin without HtrA4, incubated at 4 °C or 37 °C for 6 h. The resulting products were analysed by Western blot for VE-cadherin with a mouse anti-VE-cadherin antibody (1 µg/ml, clone TEA1/31; #PN1597, Beckman Coulter, Marseille, France). The experiment was repeated three times.

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2.2. Cell culture

HUVECs (#CRL-1730, ATCC, MD, USA) were maintained in EGM-2MV BulletKit media (EGM-2, Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Scoresby, VIC, Australia) and 1% antibiotics (Thermo Fisher Scientific). Cells were treated for 24 h in 24-well plates (for Western blot) or on 10-mm glass coverslips placed in 48-well plates (for immunofluorescence) with vehicle control or HtrA4 of 1.5 and 3.0 $\mu\text{g/ml}$ (representing the median and highest levels found in early-onset PE. The median and highest levels found in non-PE sera were 0.5 and 1.0 $\mu\text{g/ml}$ respectively).

2.3. Western blot analysis

VE-cadherin in HUVEC lysates (20 μg) was analysed by Western blot (10% SDS-PAGE), using the mouse anti-VE-cadherin antibody described above (1 $\mu\text{g/ml}$) and a HRP-conjugated donkey anti-mouse antibody (1:2000; #P0447, Dako, CA, USA). Loading control was assessed by an HRP-conjugated β -actin antibody (1:2000; Cell Signalling Technology). Densitometric analysis was performed using FIJI software (NIH, Maryland, USA) from three independent experiments ($n = 3$).

2.4. Immunofluorescence

Immunofluorescence was performed as previously described [11], using the mouse anti-VE-cadherin antibody described above (5 $\mu\text{g/ml}$) and a donkey anti-mouse Alexa Flour 488-conjugated antibody (1:200; #A11029, Molecular Probes, VIC, Australia). Cells were also stained with phalloidin (1:40; #A34044, Invitrogen) and DAPI (Sigma Aldrich). Staining was analysed with an Olympus fluorescent microscope (#Model BX53) and Cell Sens Dimension software (Olympus, VIC, Australia). Each experiment was independently conducted three times ($n = 3$).

2.5. Statistical analysis

Statistical analyses used GraphPad Prism (V.7, GraphPad Software Inc., CA, USA), and one-way ANOVA followed by Tukey's and Dunnett's post-hoc tests; $p < 0.05$ was considered significant.

3. Results and discussion

We first determined whether HtrA4 could cleave VE-cadherin *in vitro*. Recombinant VE-cadherin was incubated with HtrA4 for 30 min, 2 and 6 h respectively, and the products were analysed by Western blot (Fig. 1A). A single band corresponding to recombinant VE-cadherin was detected in all samples. No other bands were detected likely because they were too small in size and/or not recognised by the antibody. VE-cadherin remained stable at 37 °C in controls without HtrA4 but was clearly reduced when HtrA4 was present (Fig. 1A). HtrA4 cleavage of VE-cadherin was greatest at 6 h of incubation (Fig. 1A).

We next determined if HtrA4 could cleave VE-cadherin in HUVECs as an endothelial model. Cells were treated for 24 h with vehicle control, 1.5 $\mu\text{g/ml}$ or 3.0 $\mu\text{g/ml}$ HtrA4, and VE-cadherin was analysed by Western blot. A single band at the expected size of full length VE-cadherin was detected in all samples. However, VE-cadherin levels were gradually reduced by increasing doses of HtrA4 (Fig. 1B). Densitometric analysis showed that VE-cadherin was reduced by 47% ($p \leq 0.05$) and 61% ($p \leq 0.01$) by 1.5 and 3.0 $\mu\text{g/ml}$ of HtrA4 respectively (Fig. 1C). These results confirmed that HtrA4 dose-dependently reduced VE-cadherin in HUVECs.

Immunofluorescence was performed to visualize the effect of HtrA4 on VE-cadherin localisation and cell junctional integrity. Vehicle control treated HUVECs showed strong VE-cadherin staining on well-defined cell borders (Fig. 2). Cells treated with 1.5 $\mu\text{g/ml}$ HtrA4 showed thinner or discontinuous VE-cadherin staining around cells, whereas

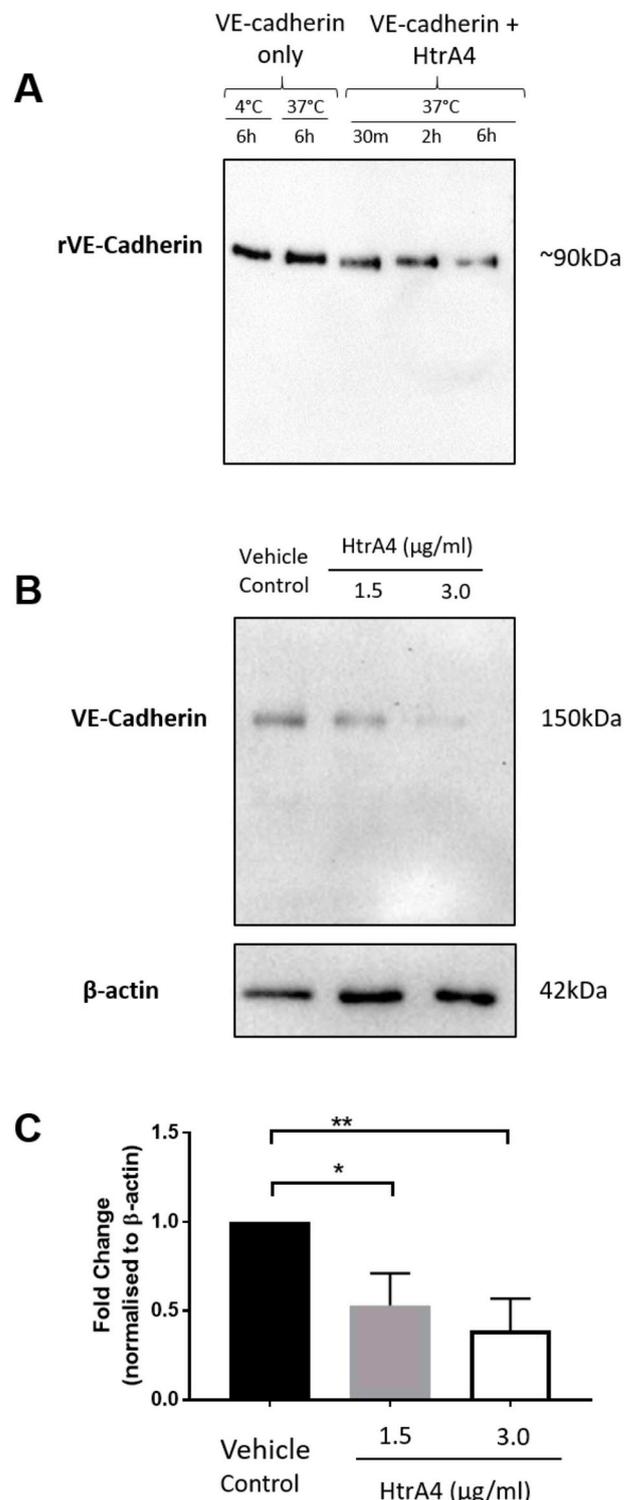


Fig. 1. Western blot analysis of VE-cadherin. (A) Recombinant VE-cadherin (rVE-cadherin, 100 ng) was incubated with recombinant HtrA4 (1 ng) for 30 min, 2 h and 6 h at 37 °C, or without HtrA4 (controls) for 6 h at 4 °C or 37 °C. The resulting products were analysed for VE-cadherin by Western blot. (B) HUVECs were treated with vehicle control, 1.5 $\mu\text{g/ml}$ or 3.0 $\mu\text{g/ml}$ HtrA4 for 24 h and total cell lysates were analysed by Western blot for VE-cadherin and β -actin. (C) Densitometric quantification of VE-cadherin in (B). Data (mean \pm SD) was normalized to β -actin and expressed as fold changes relative to the vehicle control. * $p \leq 0.05$, ** $p \leq 0.01$, $n = 3$.

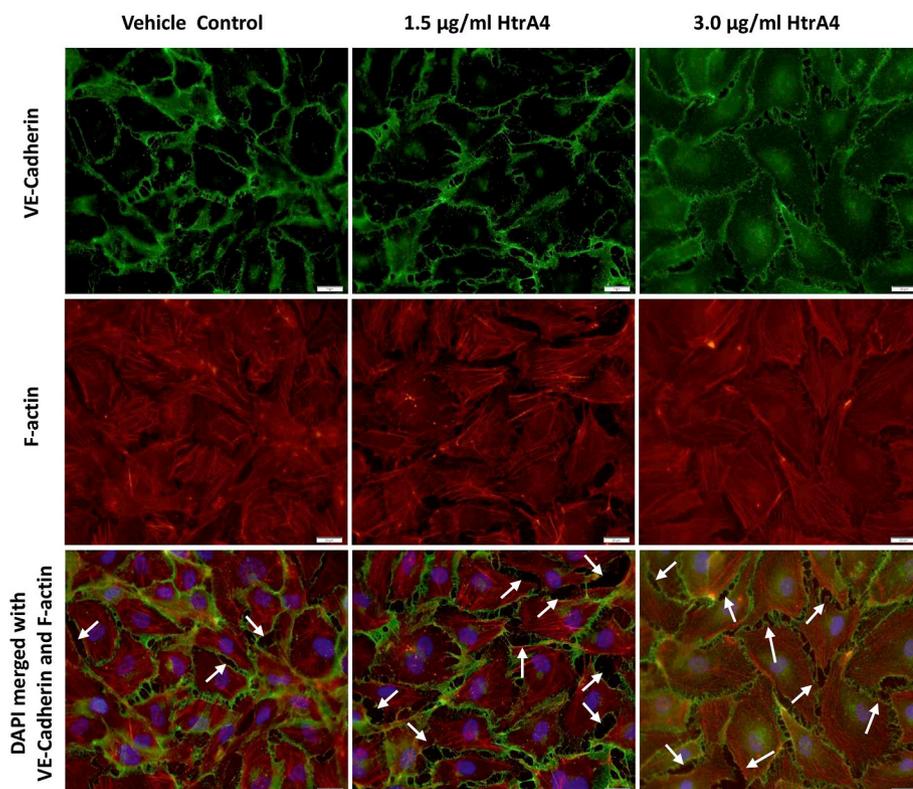


Fig. 2. Immunofluorescence of VE-cadherin in HUVECs. HUVECs were treated with vehicle control, 1.5 µg/ml or 3.0 µg/ml HtrA4 for 24 h, and immune-stained for VE-cadherin (green). They were also stained with phalloidin for F-actin (red) and DAPI for nuclei (blue). Representative images at 60x magnification from $n = 3$ experiments are shown. Scale bar = 20 µm. Arrows, intercellular gaps. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

those treated with 3.0 µg/ml displayed markedly reduced VE-cadherin on cell borders but increased staining in the cytoplasm/nuclei. Compared to controls, cells treated with both doses of HtrA4 showed more frequent and larger intercellular gaps (arrows, Fig. 2). Cell morphology was altered in cells treated with 3.0 µg/ml HtrA4. They became flatter and larger, and F-actin fibres were more parallel to cell membranes (Fig. 2).

These data are consistent with HtrA4 cleaving VE-cadherin in HUVECs to disrupt cell contacts and increase permeability [11]. Collectively, this suggests that HtrA4 may be a key placenta-derived protease that disrupts the maternal endothelium through cleaving VE-cadherin.

Conflicts of interest

All authors declare no conflict of interest.

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