Caveolin-1 opens endothelial cell junctions by targeting catenins

Romy Kronstein¹, Jochen Seebach², Sylvia Großklaus¹, Carsten Minten³, Britta Engelhardt³, Marek Drab⁴, Stefan Liebner⁵, Yvan Arsenijevic⁶, Abdallah Abu Taha², Tatiana Afanasieva¹, and Hans-Joachim Schnittler²*

¹Institute of Physiology, Medical Faculty of the TU-Dresden, Dresden, Germany; ²Institute of Anatomy and Vascular Biology, Vesaliusweg 2-4, Westfälische Wilhelms-University of Münster, Münster, Germany; ³Theodor Kocher Institute, University of Bern, Bern, Switzerland; ⁴Institute of Immunology and Experimental Therapy, Wroclaw, Poland; ⁵Institute of Neurology, Johann Wolfgang Goethe-University Frankfurt, Frankfurt, Germany; and ⁶Jules Gonin Eye Hospital, University of Lausanne, Lausanne, Switzerland

Received 8 March 2011; revised 21 September 2011; accepted 26 September 2011; online publish-ahead-of-print 29 September 2011

Time for primary review: 30 days

Aims

A fundamental phenomenon in inflammation is the loss of endothelial barrier function, in which the opening of endothelial cell junctions plays a central role. However, the molecular mechanisms that ultimately open the cell junctions are largely unknown.

Methods and results

Impedance spectroscopy, biochemistry, and morphology were used to investigate the role of caveolin-1 in the regulation of thrombin-induced opening of cell junctions in cultured human and mouse endothelial cells. Here, we demonstrate that the vascular endothelial (VE) cadherin/catenin complex targets caveolin-1 to endothelial cell junctions. Association of caveolin-1 with VE-cadherin/catenin complexes is essential for the barrier function decrease in response to the pro-inflammatory mediator thrombin, which causes a reorganization of the complex in a rope ladder-like pattern accompanied by a loss of junction-associated actin filaments. Mechanistically, we show that in response to thrombin stimulation the protease-activated receptor 1 (PAR-1) causes phosphorylation of caveolin-1, which increasingly associates with β- and γ-catenin. Consequently, the association of β- and γ-catenin with VE-cadherin is weakened, thus allowing junction reorganization and a decrease in barrier function. Thrombin-induced opening of cell junctions is lost in caveolin-1-knockout endothelial cells and after expression of a Y/F-caveolin-1 mutant but is completely reconstituted after expression of wild-type caveolin-1.

Conclusion

Our results highlight the pivotal role of caveolin-1 in VE-cadherin-mediated cell adhesion via catenins and, in turn, in barrier function regulation.

Keywords

β-catenin • Caveolin-1 • Impedance spectroscopy • VE-cadherin • Permeability

1. Introduction

Vascular endothelial (VE) cells form a barrier that controls the exchange of fluid and solutes between the vasculature and the interstitial tissue. The calcium-dependent VE-cadherin, and its associated catenins, control cell–cell adhesion, and paracellular barrier function and are important for the formation of tight junction complexes.¹² The VE-cadherin/catenin complex can be found in all endothelial cells and represents the predominant structure in endothelial junctions of postcapillary venules, a location where cell junctions preferentially open during inflammation and leukocyte diapedesis.¹² VE-cadherin has a carboxy-terminal domain that binds β-catenin, γ-catenin and p120ctn.¹² Both β- and γ-catenins bind α-catenin, which was previously suggested to connect the complex directly to actin filaments.¹² However, this concept was challenged by data showing that α-catenin is an allosteric molecule incapable of binding to both β-catenin and actin filaments at the same time.³ Recently, the epithelial protein lost in neoplasms (EPLIN) was shown to bind both actin filaments and α-catenin in epithelial cells,⁴ indicating that the α-catenin/EPLIN protein-chain might bridge the gap between cadherins and actin filaments. We were able to confirm that endothelial cells of human and mouse origin also express ELPIN (Taha and Schnittler, unpublished data). However, it is generally accepted that the VE-cadherin/catenin...
complex is functionally linked to actin filaments\textsuperscript{1,2} and that this interaction is critical in junction regulation.

Transient increases in paracellular permeability in response to activation of PAR-1 (proteinase-activated receptor-1) by thrombin cause redistribution of VE-cadherin and catenins from a continuous band into a discontinuous streak-like pattern with loss of junction-associated actin filaments and stress fibre formation. This process involves the activation of Rho-GTPases, the kinases P13K, src, and PKC, actin/myosin contractility, myosin light chain kinases and phosphatases, and the tyrosine phosphatase VEPTP (for review see).\textsuperscript{1,2} In contrast, tightening of cell junctions depends on VE-cadherin clustering accompanied by recruitment of junction-associated actin filaments via rac-1.\textsuperscript{5} Although a number of relevant mechanisms have been identified, the causal mechanism that ultimately induces opening of endothelial cell junctions in inflammation (e.g. after thrombin administration) is still unknown.

Many studies have provided compelling evidence that caveolin-1, the main scaffolding protein of caveolae,\textsuperscript{6} binds to signalling molecules\textsuperscript{7} and is involved in regulating endothelial permeability, angiogenesis,\textsuperscript{8–10} and leucocyte diapedesis.\textsuperscript{11} Caveolin-1 is composed of a lipophilic, hairpin-shaped, helical sequence embedded in the inner leaflet of the plasma membrane with both N- and C-terminal cytoplasmic domains. The N-terminus binds to many signalling molecules, including endothelial nitric oxide synthase (eNOS), src kinase, PKC\(\alpha\), and G-proteins and is required for caveolin multimerization.\textsuperscript{7} Previous studies on the role of caveolin-1 in endothelial permeability have reported both positive\textsuperscript{10,12,13} and negative\textsuperscript{8,14} effects. Caveolin-1 can be precipitated with VE-cadherin and E-cadherin\textsuperscript{13,11} and interacts with β-catenin in zebrafish.\textsuperscript{15} Thus, caveolin-1 might have a more general role in regulating cell junctions, but its molecular regulation of endothelial cell adhesion and barrier function needs to be defined. Here, we show that localization of caveolin-1 to endothelial cell junctions depends on the VE-cadherin/catenin complexes and that thrombin-induced increase in paracellular permeability is phosphocaveolin-1 dependent.

2. Methods

2.1 Antibodies and reagents

The antibodies and reagents used and their sources are listed in Supplementary material online, (SI-6).

2.2 Primary endothelial cell cultures, Chinese hamster ovary cells, endothelioma cell lines, immunofluorescence microscopy, and transendothelial electrical resistance

Human umbilical cord vein endothelial cells (HUVEC) were isolated from anonymized donors as described\textsuperscript{17} according to the principles outlined in the Declaration of Helsinki; this was approved by the ethics boards of TU-Dresden and the WW-University of Münster (EK 203112005 and 2009–537-F-S, respectively). Cells of passage 1 were used for experiments. Wild-type CHO (Chinese hamster ovary) cells and VE-cadherin-expressing CHO cells were cultured as described elsewhere.\textsuperscript{18} Caveolin-1-deficient and wild-type endotheloma cell lines were established from hearts of caveolin-1-deficient newborn mice\textsuperscript{19} and wild-type mice, respectively, as previously described.\textsuperscript{20} Animals were sacrificed by cervical dislocation in accordance to the German animal protection law and to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85–23, revised 1996). A β-catenin-deficient cell line was a kind gift from Elisabetta Dejana, Milano, and cultured as described.\textsuperscript{21} Immunolabeling was carried out as previously described\textsuperscript{17} and detected using a laser-scanning microscope (LSM 510 Zeiss, Jena, Germany). Transendothelial electrical resistance (TER) of endothelial cell cultures was determined from impedance spectroscopy data.\textsuperscript{22} Endothelioma cell cultures having a TER >3 Ec mh\textsuperscript{2} a value sufficient to evaluate endothelial barrier function by impedance spectroscopy, were used for experiments.

2.3 Generation of recombinant glutathione-S-transferase-tagged β-catenin and VE-cadherin cytoplasmic domain

Recombinant murine glutathione-S-transferase (GST)–β-catenin was expressed in E. coli BL21 using the pGEX 2T-1 expression vector (kindly provided by William Weis and S. Pokutta, Stanford, USA) and purified as previously described.\textsuperscript{22} The recombinant cytoplasmic domain of GST-VE-cadherin (GST-VE-cad\textsubscript{cyto}) was expressed in E. coli strain BL21 using the pGEX 4T-1 expression vector (kindly provided by D. Vestweber, Münster, Germany). GST-VE-cad\textsubscript{cyto} was purified using a GSTrap\textsuperscript{23} FF glutathione-agarose column (GE-Healthcare, Uppsala, Sweden) according to the manufacturers’ instruction manual.

2.4 GST pull-down assays

Either GST-tagged β-catenin or VE-cad\textsubscript{cyto} (65 nM) was bound for 1 h at 4 °C to glutathione sepharose in binding buffer (20 mM Tris/HCL pH 7.4, 150 mM NaCl 1% Triton-X-100) and washed three times in 1 mL of phosphate-buffered saline containing 0.5% Triton X-100, 1 mM dithiothreitol (DTT) and 1 mM PMSF (washing buffer). HUVEC cell cultures (7 × 10\textsuperscript{5} cells) were lysed for 15 min at 4 °C in lysis buffer (50 mM Tris/HCL, pH 7.6, 150 mM NaCl 1% Triton X-100, 1 mM PMSF, 5 mM DTT, and protease inhibitors), and pelleted at 10 000 g for 5 min. Precleared supernatants were subsequently adjusted to 650 μg total protein/mL and a total volume of 800 μL, GST-β-catenin- or GST-VE-cad\textsubscript{cyto}-loaded glutathione beads were added to HUVEC cell lysates (7 × 10\textsuperscript{6} cells) for 2 h at 4 °C. After three washes using washing buffer, the samples were lysed in SDS-sample buffer and analysed by SDS–PAGE and western blotting.

2.5 DNA plasmids, constructs, and production of lentiviral vectors

Mouse caveolin-1\textsuperscript{22} was cloned into the lentiviral vector pFUGW via the BamHI and EcoRI cloning sites following PCR amplification with forward primer 1 (5′-GATGATCACGATGTCTCAGGCGCAATACGTG-3′, BamHI) and reverse primer 2 (5′-GGGAAATCTCTTCCTCTTGTCGGTCG-3′, EcoRI). Cav-1-Y14F was generated by PCR amplification of fragment 1 using forward primer 3 (5′-GGGAAATCTCTTCCTCTTGTCGGTCG-3′, EcoRI) and reverse primer 1 and reverse primer 2 (5′-GGGAAATCTCTTCCTCTTGTCGGTCG-3′, EcoRI). The two fragments were gel-purified and annealed, and PCR was performed using primers 1 and 2. Purified PCR products were digested using BamHI and EcoRI and then cloned into the lentiviral vector pFUGW using the same cloning sites. Full-length mouse β-catenin\textsuperscript{24} was amplified by PCR from the pCS2+ vector using the forward primer (5′-GGGAAATCTCTTCCTCTTGTCGGTCG-3′, base exchange A/T is shown in bold) and reverse primer 2 (5′-GGGAAATCTCTTCCTCTTGTCGGTCG-3′, EcoRI). The purified PCR product was ligated into the pCMV-EGFP vector (Clontech, Mountain View, USA) and packaged in 293FT cells using the reverse primer (5′-GGGAAATCTCTTCCTCTTGTCGGTCG-3′). The recombinant GFP product was verified by sequencing. Lentiviruses were produced by triple transfection of 293 T cells with pFUGW carrying the gene of interest, packaging vectors, and pMD2G carrying the VSV glycoprotein as described elsewhere.\textsuperscript{25} Viral stocks were titrated by HIV-1 ELISA according to the manufacturers’ directions (Zeptometrix Cooperation, Buffalo, USA). Gene transduction into endothelioma cells was performed using 10 TU/cell. Gene transduction efficiency was >90%, as scored by the number of...
caveolin-1-positive cells. Lentivirus-mediated gene transfer was non-toxic as tested by determination of endothelial barrier function, growth, and morphology. Protein expression was verified by quantitative western blot analysis and immunofluorescence microscopy, as indicated.

2.6 Analysis of tyrosine phosphorylation, immunoprecipitation, and western blotting

Tyrosine phosphorylation assay and quantitation was performed as described elsewhere and outlined in the Supplementary material online.

2.7 Quantification of western blots, cell junction proteins, and statistical analyses

Quantitative western blots using total cell extracts (Figure 1) were performed under standard conditions; prior to gel loading, total protein was determined in SDS-dissolved samples, and defined amounts of protein were loaded on each lane. Confluent cultures of HUVEC were pooled (125 cm² total) and subsequently divided in five different samples, each used for quantitative immunoprecipitation (IP) of VE-cadherin, α-, β-, or γ-catenin, which yielded varying amounts of caveolin-1 and associated junction proteins (Figure 1A and B). IP of β-catenin and its homologue γ-catenin yielded the highest amount of caveolin-1 (~10% of the total), whereas IP of VE-cadherin yielded much less (Figure 1A and B). These interactions were specific as IP of neither zonula occludens protein-1 nor mouse IgG2a co-precipitated caveolin-1, VE-cadherin, or catenins (Figure 1A and B). These results prompted us to test the ability

Figure 1 Caveolin-1 is found in a complex with junction molecules in endothelial cells. (A and B) Densitometry-based quantitative western blot analyses of IP of VE-cadherin, α-catenin, β-catenin, ZO-1, γ-catenin, PECAM-1, or unspecific mouse IgG2a from same amounts and volume of Triton X-100 soluble cell extracts. (C) GST-β-catenin, or the GST-coupled cytoplasmic domain of VE-cadherin (GST-VE-cadcyto) was immobilized to glutathione-S-sepharose beads and incubated with Triton X-100-soluble HUVEC cell lysates and probed by western blotting as indicated. Total cell lysates served as a positive control. *P < 0.05; **P < 0.005; ***P < 0.0005.

3. Results

3.1 Caveolin-1 associates with the VE-cadherin/catenin complex

We first determined that caveolin-1 is an integral component of the VE-cadherin/catenin complex in HUVECs, as indicated by IP of VE-cadherin, α-, β-, or γ-catenin, which yielded varying amounts of caveolin-1 and associated junction proteins (Figure 1A and B). IP of β-catenin and its homologue γ-catenin yielded the highest amount of caveolin-1 (~10% of the total), whereas IP of VE-cadherin yielded much less (Figure 1A and B). These interactions were specific as IP of neither zonula occludens protein-1 nor mouse IgG2a co-precipitated caveolin-1, VE-cadherin, or catenins (Figure 1A and B). These results prompted us to test the ability
of GST-β-catenin and the GST-coupled cytoplasmic domain of VE-cadherin (GST-VE-cad_{cyto}) to precipitate caveolin-1 from endothelial cell extracts. Indeed, GST-β-catenin, but not GST-VE-cad_{cyto} or GST-glutathione-sepharose, pulled down caveolin-1 (Figure 1C). These data indicate that caveolin-1 associates with the VE-cadherin/catenin complex in endothelial cells and requires β-catenin for interaction. Since PECAM-1 was shown to be associated with both α/β integrin27 and β-catenin,28 we expected caveolin-1 in PECAM-1 immunoprecipitates. Indeed, IP of PECAM-1 yielded a fraction containing both β-catenin and caveolin-1 (Figure 1A and B).

### 3.2 Recruitment of caveolin-1 to cell junctions depends on the VE-cadherin/catenin complex

Immunostaining of confluent cultures of HUVEC showed that a fraction of caveolin-1 localized along the cell junctions and appeared superimposed with VE-cadherin, catenins, and PECAM-1 (Supplementary material online, SI-1). To determine whether VE-cadherin and/or the catenins, in particular β-catenin, are important for the junction localization of caveolin-1, we analysed the distribution of caveolin-1 in two different cell systems: CHO cells and β-catenin-knockout endothelium cells.21 CHO cells endogenously express caveolin-1 and small amounts of β-catenin, but not α-catenin, γ-catenin, PECAM-1, VE-cadherin, or other classical cadherins (Figure 2A and B; Supplementary material online, SI-2). In wild-type CHO cells neither β-catenin nor caveolin-1 was localized to the cell junctions (Figure 2A, a–d), while expression of VE-cadherin recruited both the bulk of the β-catenin and a fraction of the caveolin-1 (Figure 2A, e–h). Since VE-cadherin expression also induced up-regulation of both β-catenin and α-catenin,29 but not caveolin-1 (Figure 2B), we further investigated whether caveolin-1 recruitment to cell junctions was simply related to the increased amount of β-catenin in VE-cadherin-expressing CHO cells. When overexpressed in CHO cells (Figure 2C), β-catenin was predominantly localized in the cytosol and caveolin-1 was not seen at cell junctions (Figure 2A; i–l). Consistently, we found by IP that association of caveolin-1 with β-catenin was high in VE-cadherin-expressing CHO cells, much lower in β-catenin-overexpressing CHO cells, and negligible in wild-type CHO cells (Figure 2D). Since caveolin-1 is a component of a number of subcellular domains its interaction with β-catenin requires spatial proximity at junctions as seen in VE-cadherin-expressing CHO (Figure 2A; e–l) and endothelial cells (Figures 3; Supplementary material online, SI-1). In addition, we used β-catenin-knockout endothelial cells21 that still expresses VE-cadherin, α- and γ-catenin, and caveolin-1 (Supplementary material online, SI-3). Since immunoprecipitates of γ-catenin also yield caveolin-1 (Figure 1), we found a small fraction of caveolin-1 at cell junctions in β-catenin-knockout endothelial cells, as expected (Figure 3A). Re-expression of β-catenin in β-catenin-knockout endothelial cells increased the junction localization of caveolin-1 by a factor of 2.5 (Figure 3A and B). This amount of caveolin-1 is comparable with the amount of caveolin-1 found in wild-type endothelial cells of both murine and human origin. Thus, the VE-cadherin/catenin complex is able to recruit caveolin-1 to cell junctions and β-catenin greatly facilitates this effect.

### 3.3 Thrombin stimulation increases formation of caveolin-1 complexes with β- and γ-catenin and decreases association with VE-cadherin

The functional relevance of caveolin-1 complex formation with VE-cadherin and catenins was tested by stimulation with thrombin, a serine protease that cleaves the PAR-1 receptor and consequently increases paracellular permeability.30 Impedance spectroscopy was used to determine TER.5 One unit of thrombin was found to transiently down-regulate TER to 70% of baseline values; the most significant effect in HUVEC was seen 10–25 min post-stimulation, with full recovery after 1 to 2 h (Figure 4A). Furthermore, standardized IP of junction components revealed that thrombin stimulation decreased the association of caveolin-1 with VE-cadherin to about one-third after 20 min; this returned to pre-stimulation levels within 120 min (Figure 4B and C). In contrast, thrombin stimulation increased caveolin-1 association with both β-catenin- and γ-catenin by two-fold in Triton X-100-soluble cell extracts as shown by IP of the respective catenins (Figure 4B, D, and E). We performed IPs of catenins instead of caveolin-1 as catenins appear to be target proteins of caveolin-1 at cell junctions. Consistently, a fraction of β-catenin dissociated from VE-cadherin after thrombin stimulation, as assayed by VE-cadherin IP followed by β-catenin detection in Triton X-100 cell extracts. This effect became increasingly apparent when the β-catenin/VE-cadherin ratio was calculated (Figure 4F and G). These effects were highly specific, since the amount of caveolin-1 bound to α-catenin and PECAM-1 remained unchanged in response to thrombin stimulation (Figure 4B, H, and I).

### 3.4 Remodelling of the VE-cadherin/catenin complex during thrombin-induced opening and reconstitution of cell junctions

Consistent with the thrombin-induced TER-minimum VE-cadherin (Figure 5A), β-catenin (Figure 5B and C), and γ-catenin (not shown) underwent remodelling from a continuous to an interrupted and rope ladder-like pattern (Figure 5). A fraction of caveolin-1 localized at the cell junctions under control conditions (Figure 5A) and underwent remodelling in accordance with the redistribution of the VE-cadherin/catenin complex into a rope ladder-like pattern, as shown for β-catenin and caveolin-1 (Figure 5C). This remodelling was accompanied by the characteristic loss of junction-associated actin filaments and the development of stress fibres (Supplementary material online, SI-4). During TER-recovery both caveolin-1 and the VE-cadherin/catenin complex reappeared at the junctions after 120 min, as shown for VE-cadherin and β-catenin (Figure 5A and D). The thrombin-induced junction reorganization was also seen after stimulation of endothelial cells with the PAR-1-specific PAR-1-activated peptide (PAR-1AP; not shown). These data further support the concept that the impact of caveolin-1 on junction regulation occurs via interaction with the VE-cadherin/catenin complex.

### 3.5 Caveolin-1 is critical for the transient breakdown of paracellular barrier function

Since caveolin-1 can be activated by phosphorylation at tyrosine 14,31 we found a transient increase in phosphocaveolin-1 in β-catenin IP after thrombin stimulation (Figure 6A), whereas the amount of
Figure 2  VE-cadherin/catenin complexes target caveolin-1 to cell junctions in CHO cells. Wild-type CHO cells (CHO wt), CHO cells expressing VE-cadherin (CHO-VE-cad), and CHO cells overexpressing β-catenin (CHO + β-cat) were immunolabelled for (A) VE-cadherin (panels a, e and i) or triple-labelled for β-catenin, caveolin-1, and nuclear DNA (DAPI). Yellow colour indicates co-localization of β-catenin and caveolin-1 (arrows). Expression of VE-cadherin but not β-catenin increased recruitment of both β-catenin and caveolin-1 to cell junctions. Inserts indicate confocal sections in the vertical plane of the z-stacks taken at the position shown by the white lines. Stars indicate same cell in the series. Bars = 10 μm. (B) Total cell lysates (20 μg/lane) were analysed by western blotting as indicated. HUVEC, positive control. Expression of VE-cadherin CHO wild-type (wt) increased expression of β- and α-catenin. Alpha-tubulin, internal control. (C) Total cell lysates (10 μg/lane) as indicated were probed by western blotting for β-catenin. Increased degradation of β-catenin is seen only in CHO wt + β-cat cells. (D) IP of β-catenin from cell lysates as indicated was probed for either VE-cadherin or caveolin-1.
phosphorylated caveolin-1 precipitated with the VE-cadherin antibody remained unchanged (Figure 6A). To prove this concept, we generated caveolin-1-knockout endothelioma cells and expressed the wild-type caveolin-1 or caveolin-1 14Y/F (cav-1-Y/F), a mutant form of caveolin-1 that can no longer be phosphorylated at tyrosine 14 (Figure 6B). The caveolin-1-knockout endothelioma cells are shown in the Supplementary material online, SI-5. Thrombin stimulation of caveolin-12/2 endothelial cells (cav2/2 cells) did not decrease the TER, but rather increased it slightly (Figure 6C). Expression of caveolin-1 in cav2/2 cells (cav2/2 cells + cav-1) completely reconstituted the thrombin response, while expression of the caveolin-1 Y/F mutant (cav2/2 cells + cav-1Y/F) did not. Thus, cav2/2 cells + cav-1Y/F behaved like cav2/2 cells lacking caveolin-1 (Figure 6C). Under these conditions, the association of caveolin-1 with β-catenin increased in cav2/2 cells + cav-1 but not in cav2/2 cells + cav-1Y/F (Figure 6D and E). Consistent with the hypothesis that phosphocaveolin-1 mediates dissociation of β-catenin from VE-cadherin, we found decreased amounts of β-catenin in VE-cadherin IPs after thrombin application in cav2/2 cells + cav-1 but not in cav2/2 cells or cav2/2 cells + cav-1Y/F. Rather, the addition of thrombin to cav2/2 cells and cav2/2 cells + cav-1Y/F increased formation of β-catenin complexes with VE-cadherin (Figure 6F). Together, these data provide direct evidence that phosphocaveolin-1 is required to open intercellular junctions in response to thrombin by increasing the formation of complexes with catenins that dissociate from VE-cadherin.

4. Discussion
Regulation of paracellular endothelial barrier function requires a concerted interplay between cell adhesion molecules and the
Figure 4. Thrombin-induced barrier function decrease is associated with increased formation of catenin/caveolin-1 complexes that dissociate from VE-cadherin. (A) Transendothelial electrical resistance (TER) was determined by impedance spectroscopy and plotted relative to baseline TER values (TER/TER (0)) in HUVEC with and without 1 U/mL of thrombin. TER(0) values ranged between 8 and 15 Ω cm². n = 3. ***P < 0.0005. (B–I) HUVEC were treated with 1 U/mL of thrombin followed by IP of adherens junction proteins and subsequent quantitative western blotting, as indicated. Prior to IP, nuclei-free Triton X-100 cell extracts from each experimental series were adjusted for equal protein content and equal volume. A sample of 20 μL was blotted and probed for α-tubulin content for internal control and quantification of relative protein amounts of co-precipitated proteins (panels C1–E1, G1–I1). (B) Densitometry of western blots after IP with antibodies as indicated, n = 3. (C–E, H–I) One out of the three independent western blots that were used for quantification as indicated is shown. (C) Caveolin-1 dissociated transiently from the VE-cadherin/catenin complex after 20 min of thrombin stimulation, while caveolin-1 transiently increased its association with both (D and E) β-catenin and γ-catenin. (F and G) β-Catenin/VE-cadherin ratios were determined by densitometry of western blots (one out of three is seen in G) after thrombin stimulation. (H and I) Association of caveolin-1 with α-catenin and PECAM-1 remained unchanged. *P < 0.05; **P < 0.005; ***P < 0.0005.
junction-associated actin cytoskeleton. The ability of caveolin-1 to bind to and control certain regulatory molecules, such as src kinases, G-proteins, and eNOS, as well as its ability to modulate calcium signalling and permeability\textsuperscript{6,8,19,32,13} indicates a critical role in junction dynamics and integrity. Here, we aimed to find novel clues as to how caveolin-1 controls cell adhesion and barrier function in endothelial cells. To this end, we demonstrated a caveolin-1-controlled mechanism that is directly linked to VE-cadherin-mediated cell adhesion in response to thrombin stimulation.

The most significant findings of this study are (i) that caveolin-1 associates with and is targeted by the VE-cadherin/catenin complex to endothelial cell junctions and (ii) that phosphocaveolin-1 is critical for the opening of endothelial cell junctions in response to thrombin stimulation. Association of catenins with caveolin-1 was demonstrated by IPs, GST-pull-down assays (Figure 1), and expression studies using CHO cells (Figure 2) and a β-catenin-knockout cell line (Figure 3). The data are in line with other reports showing that VE-cadherin and E-cadherin are associated with caveolin-1\textsuperscript{13,15} as well as the identification in zebrafish of a binding motif that mediates the interaction of caveolin-1 with β-catenin.\textsuperscript{16} Thus, a direct interaction of mammalian caveolin-1 with mammalian β-catenin might occur, but this has to be tested in the future.

The functional relevance of our findings was further demonstrated in primary cultures of HUVEC and a caveolin-1-knockout endothelial cell line after stimulation with thrombin. Certain signalling pathways become activated by thrombin through G-protein-coupled PAR-1. This includes activation of Rho-GTPases, non-receptor tyrosine kinases such as Pyk2 and src-kinase, myosin light chain kinases and phosphatases,\textsuperscript{33,34} and actin/myosin filament sliding mechanisms.\textsuperscript{35,36} Furthermore, caveolin-1 becomes tyrosine phosphorylated by src-kinases,\textsuperscript{13} a process that has also been described to be critical in barrier function regulation.\textsuperscript{34} Indeed, thrombin induces tyrosine phosphorylation of caveolin-1 by hitherto unknown non-receptor tyrosine kinases, which in turn increases complex formation with both β-catenin and γ-catenin and weakens binding to VE-cadherin. This is reflected in the decreased amounts of both β-catenin and caveolin-1 that were precipitated from soluble Triton X-100 cell extracts with VE-cadherin and the increased amounts of caveolin-1 that were

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Thrombin-induced reorganization of junction molecules. Immunolabelling of HUVEC after treatment with either 1 U/mL thrombin or buffer as indicated. (A–D) Thrombin stimulation induced a transient redistribution of VE-cadherin, β-catenin, and caveolin-1 into a rope ladder-like pattern within 15 min that recovered after 120 min. Boxes indicate areas of higher magnification. Arrows point to cell junctions. Under control conditions (B) a fraction of caveolin-1 co-localized at cell junctions and with a fraction of β-catenin (yellow staining, arrowheads). Thrombin administration dissociated caveolin-1 from cell junctions and (C) colocalized with rope ladder-like β-catenin staining (arrows). (D) Within 120 min both caveolin-1 and β-catenin reappeared at junctions as under control conditions. Panels on the right-hand side show magnification of the respective boxes. Nuclei are stained with DAPI. Scale bars = 20 μm.}
\end{figure}
Phosphocaveolin-1 is required to open cell junctions in response to thrombin. (A) Quantitative western blot of phosphocaveolin-1 using a Y-14 phosphocaveolin-1-specific antibody after IP of adherens junction molecules from HUVEC treated with thrombin as indicated. Phosphocaveolin-1 was transiently increased in the β-catenin IP, whereas the amount of phosphocaveolin-1 that associated with VE-cadherin remained unchanged. Pervanadate (PV) served as a positive control. *P < 0.05; **P < 0.005; ***P < 0.0005. (B) Expression of caveolin-1 and the 14 Y/F-caveolin-1-mutant in cav-1−/− endothelial cells by lentiviral gene transfer. Alpha-tubulin served as internal protein loading control. (C) TER of endothelioma cells as indicated in response to thrombin. Only caveolin-1-expressing cells showed the characteristic decrease in TER. (D) Quantitative western blotting of β-catenin IP from endothelioma cells as indicated. (E) Immunoprecipitates of β-catenin from indicated cell lines were probed for caveolin-1 after thrombin stimulation. (F) Western blot of caveolin-1 from total Triton X-100 cell extracts as indicated. α-tubulin served as a further loading control. (F) IP of VE-cadherin from indicated cell lines was probed for β-catenin after thrombin stimulation. One representative blot from three independent experiments is shown. Quantitative analysis of three independent experiments is shown. *P < 0.05; **P < 0.005.

R. Kronstein et al. 138
precipitated with β- and γ-catenin. The non-ionic detergent Triton X-100 dissociates protein complexes only when the association is weak, and it is reasonable to assume that the β-catenin-/phosphocaveolin-1 complex is dissociated from VE-cadherin after Triton X-100 extraction. These results are also consistent with the localization of caveolin-1 and β-catenin in a rope ladder-like pattern after thrombin stimulation (Figure 5C). While histamine stimulation has been shown to increase the association of β-catenin with PECAM-1, a mechanism that might also reduce the association of β-catenin with VE-cadherin, the interaction of PECAM-1 with caveolin-1 seems to be of less importance after thrombin stimulation (Figure 4B). Another possible role of caveolin-1 in the regulation of endothelial barrier function was recently reported in the form of hydrogen peroxide-induced dissociation of β-catenin and caveolin-1 in rat lung microvascular endothelial cells. 

Caveolin-1 seems to be of less importance after thrombin stimulation (Figure 5C). While histamine stimulation has been shown to increase the association of β-catenin with VE-cadherin, the interaction of PECAM-1 with caveolin-1 seems to be of less importance after thrombin stimulation (Figure 4B). Another possible role of caveolin-1 in the regulation of endothelial barrier function was recently reported in the form of hydrogen peroxide-induced dissociation of β-catenin and caveolin-1 in rat lung microvascular endothelial cells. 

Hydrogen peroxide is an important but also an unspecific stimulus that leads to increased endothelial permeability, and a different mechanism utilizing caveolin-1 seems to be involved. This shows the existence of different mechanisms that seem to depend on the respective stimuli but might also depend on extraction buffers and cell type. However, both studies (and the present study) point to the critical importance of caveolin-1 in barrier function regulation.

Catenins occupy a strategically important position at the adherens junctions since they link VE-cadherin to the cytoskeleton. Weakening of this interaction by phosphocaveolin-1, together with Rho activation in response to thrombin stimulation, might facilitate uncoupling of the VE-cadherin/catenin complex from the junction-associated actin cytoskeleton, leading to the formation of stress fibres and the development of contractile forces. In addition, caveolin-1 interacts with the actin filament cross-linking protein filamin, and this interaction might be important in uncoupling the VE-cadherin/catenin complex from the actin cytoskeleton. Previous work showed lateral clustering of the VE-cadherin/catenin complex and a rac-1-dependent recruitment of actin filaments to adherens junctions in response to physiological shear stress, a stimulus that tightens endothelial cell junctions. Thus, thrombin-induced weakening of β- and γ-catenin binding to VE-cadherin might also lead to reduced lateral clustering of the VE-cadherin/catenin complex. However, whether and to what extent lateral clustering contributes to the regulation of the VE-cadherin/catenin complex and its connection to the actin cytoskeleton during inflammation has yet to be determined.

Here, we show that PAR-1 activation leads to phosphorylation of caveolin-1, which in turn is essential to open endothelial cell junctions but is independent of the signalling that leads to ERK1/2 phosphorylation. Thus, PAR-1-mediated activation of caveolin-1 turns on multiple pathways that appear to be independent of each other. The data are in line with β-catenin-mediated, thrombin-induced long-term stimulation of gene expression, and support the concept that β- and γ-catenin are targets in the opening of cell junctions. Other stimuli such as histamine also target β-catenin, and γ-catenin was shown to be targeted by VE phosphotyrosine phosphatase during leukocyte diapedesis. We assume that caveolin-1 plays a role in these processes as well, but this has to be investigated in a separate study.

The role of caveolin-1 in the regulation of endothelial barrier function has also been studied using a siRNA approach. Depletion of caveolin-1 increases constitutive endothelial permeability and reduces VE-cadherin and β-catenin levels (Supplementary material online, SI-5). However, the caveolin-1-knockout cells still formed a barrier (Supplementary material online, SI-5) and responded to thrombin with a slight increase in barrier function, as seen by an increase in TER. This phenomenon demonstrated a counter-regulatory mechanism that is activated in parallel by thrombin but is independent of caveolin-1. In contrast, caveolin-1 is essential in the opening of cell junctions in response to thrombin. Thrombin increases paracellular permeability in a process requiring the phosphorylation of caveolin-1 at tyrosine 14.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

We thank Christine Mund, Antje Messer, and Annelie Ahle for excellent technical assistance. Teymuras Kurzchalia kindly provided us with breeding pairs of caveolin-1 knockout mice. William Weis and Sabine Pokutta (Stanford, USA) generously provided the plasmids for recombinant GST-β-catenin, and Dietmar Vestweber (Münster, Germany) provided the plasmids for the generation of recombinant GST-VE-cadherin cytoplasmic domain and the VE-cadherin-expressing CHO cells. Elisabetta Dejana kindly provided the mouse β-catenin endothelioma cells and Rolf Klemmer (Tübingen, Germany) the mouse β-catenin cDNA. We are grateful to Michele Solimena, Kai Simons, and Rolf Jessberger for helpful discussion.

Conflict of interest: none declared.

Funding

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SCHN 430/3–3 and SCHN 430/6–1) and the CRTD Dresden.

References