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VE-cadherin and β-catenin binding dynamics during histamine-induced endothelial hyperpermeability

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Abstract

β-Catenin plays an important role in the regulation of vascular endothelial cell-cell adhesions and barrier function by linking the VE-cadherin junction complex to the cytoskeleton. The purpose of this study was to evaluate the effect of β-catenin and VE-cadherin interactions on endothelial permeability during inflammatory stimulation by histamine. We first assessed the ability of a β-catenin binding polypeptide known as inhibitor of β-catenin and T cell factor (ICAT) to compete β-catenin binding to VE-cadherin in vitro. We then overexpressed recombinant FLAG-ICAT in human umbilical vein endothelial cells (HUVECs) to study its impact on endothelial barrier function controlled by cell-cell adhesions. The binding of β-catenin to VE-cadherin was quantified before and after stimulation with histamine along with measurements of transendothelial electrical resistance (TER) and apparent permeability to albumin (P_a) under the same conditions. The results showed that ICAT bound to β-catenin and competitively inhibited binding of the VE-cadherin cytoplasmic domain to β-catenin in a concentration-dependent manner. Overexpression of FLAG-ICAT in endothelial cell monolayers did not affect their basal permeability properties, as indicated by unaltered TER and P_a; however, the magnitude and duration of histamine-induced decreases in TER were significantly augmented. Likewise, the increase in P_a in the presence of histamine was exacerbated. Overexpression of FLAG-ICAT also significantly decreased the level of β-catenin-associated VE-cadherin following histamine stimulation. Taken together, these data suggest that inflammatory agents like histamine cause a transient and reversible disruption of binding between β-catenin and VE-cadherin, during which endothelial permeability is elevated.

Keywords

endothelial barrier; cell-cell junction; signal transduction; inflammation

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The Vascular Endothelial Barrier regulates the passage of fluid, solutes, and circulating cells from the blood into the surrounding tissues. The permeability property of this barrier is determined in part by the integrity of endothelial cell-cell adherens junctions. Disruption of the junctions can lead to microvascular leakage, a common and important consequence of inflammation seen in ischemia-reperfusion injury, trauma, and diabetic microvascular complications (28, 47–49). Many inflammatory stimuli have been shown to be capable of altering endothelial cell-cell adhesive interactions rendering leakage via the paracellular pathway (35, 39, 42, 46). The precise molecular mechanisms underlying the increased paracellular permeability in inflammation remain incompletely understood. Heterogeneous responses are observed among various tissue origins of endothelial cells under different types of stimulation.

Several structural and signaling molecules have been characterized for their roles in forming and regulating endothelial cell-cell junctions in the vascular wall. Among them, VE-cadherin, a transmembrane protein that comprises the adherens junction, is essential to the maintenance of microvascular barrier properties (7, 8, 18). The molecular basis underlying VE-cadherin-mediated cell-cell adhesion has been a subject of intense investigation (2, 9, 22, 28, 30, 41, 46), resulting in the identification of several catenins in the junction complex as important molecular linkage between VE-cadherin and the cytoskeleton. The β-isoform of catenin is a multifunctional protein that participates in both Wnt signaling and cell-cell adhesive interactions. In endothelial cells, β-catenin joins other catenins forming a junction complex anchored to the actin cytoskeleton, promoting cell-cell adhesions and maintaining barrier function (28, 45). We have previously shown that the expression of a recombinant VE-cadherin cytoplasmic domain construct, which competes with native VE-cadherin for β-catenin binding, increases the permeability of both endothelial cell monolayers and intact coronary venules (18).

While the aforementioned study demonstrates the importance of β-catenin/VE-cadherin binding in maintaining normal endothelial barrier function under nonstimulated conditions, other studies have identified changes in the adherens junction complex that occur in association with endothelial hyperpermeability elicited by inflammatory stimuli. For example, histamine, a typical edematogenic factor involved in acute inflammation, has been shown to increase tyrosine phosphorylation of VE-cadherin and β-catenin (3, 33). Likewise, systemic inflammation caused by thermal trauma produces a similar response in endothelial cells characteristic of junction disorganization and β-catenin redistribution (37–39). It is not clear, however, whether altered binding of VE-cadherin and β-catenin serves as a mechanism for the hyperpermeability response.

The purpose of this study was to test whether decreased binding between β-catenin and VE-cadherin contributed to histamine-induced endothelial hyperpermeability. We validated the construction and overexpression of the 9-kDa inhibitor of β-catenin and T cell factor (ICAT) as a tool to investigate the interaction between β-catenin and VE-cadherin with respect to its importance in maintaining junctional integrity. ICAT was chosen for its ability to competitively bind to free β-catenin (13, 14, 16, 36). Previous reports have indicated that ICAT overexpression does not irreversibly disrupt the β-catenin-dependent cadherin complex in cultured epithelial cells or *Xenopus* oocytes in vivo; however, ICAT

overexpression apparently reduces E-cadherin-mediated cell-cell adhesions during periods of dynamic junctional rearrangement (14,15). Intrigued by the findings in epithelial cells, we evaluated the role of ICAT overexpression on β-catenin-VE-cadherin binding dynamics and barrier function in human vascular endothelial cells.

Materials and Methods

Protein expression and purification

Total RNA was extracted from human umbilical vein endothelial cells (HUVECs). The cDNA product spanning the coding region of ICAT mRNA was amplified using RT-PCR (5′-primer: 5′-AACCGCGAGGGAGCTCCCGGGA-AGA-3′ and 3′-primer: 5′-TGCAGCTACTGCCTCCGGTCTTC-CGTCTC-3′, based on the human ICAT mRNA sequence, GenBank Accession No. AB021262). The PCR product was cloned into the pQE-30UA vector (Qiagen, Valencia, CA), with the recombinant ICAT expressed as a 5′-terminal, 6× His-tagged fusion protein. Fresh culture of *Esherichia coli* harboring the plasmid pQE30/ICAT were incubated with LB broth containing ampicillin (100 μg/ml) at 37°C for 2 h. Isopropyl-β-D-thioglactoside (IPTG) was then added to the bacterial culture at 1 μM, followed by an incubation for an additional 4 h. The culture was harvested, and cell pellet was resuspended in B-PER Reagent (Pierce, Redford, IL) for lysis. The clarified supernatant was loaded into prebalanced nickel-nitrilotriacetic acid (Ni-NTA) spin columns (Qiagen). After a wash, ICAT was eluted in a buffer containing 250 mM imidazole, and the extract was dialyzed against 20 mM Tris·HCl-buffered saline (pH 7.5) for the removal of imidazole. The His-tagged VE-cadherin cytoplasmic domain (CPD) and GST-tagged β-catenin (residues 134–664) were individually expressed in *E. coli* and purified as previously described (15, 18).

Protein binding assays

Recombinant ICAT protein immobilized on Ni-NTA agarose beads was incubated at 4°C for 4 h with HUVEC lysate. Beads were washed five times with 20 mM Tris·HCl-buffered saline (pH 7.5) containing 0.3% Triton X-100 and then boiled in a sample loading buffer. Eluted proteins were subjected to PAGE and Western blot analysis. After protein transfer, the polyvinylidene difluoride membrane (0.2 μm) was first blotted with a monoclonal antibody to the His tag (Qiagen) for the detection of His-tagged ICAT. Afterward, the membrane was stripped and reprobed with horseradish peroxidase-conjugated anti-β-catenin (BD Biosciences, Lexington, KY). For the competitive binding assay, GST-tagged β-catenin (residues 134-664) was immobilized on glutathione agarose beads (Pierce) by an incubation of the beads with β-catenin-expressing *E. coli* lysate. Binding assays were performed in 20 mM Tris·HCl buffer (pH 8.0) containing 100 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT. His-tagged VE-cadherin CPD and His-ICAT were sequentially added to GST-β-catenin-bound beads and incubated at 4°C for 2 h in a total volume of 100 μl. After centrifugation, the supernatant was removed, and beads were washed five times with wash buffer [20 mM Tris (pH 8.0), 20 mM KCl, 1 mM DTT, and 0.1% Triton X-100]. After the last wash step, beads were resuspended in 50 μl of gel loading buffer, and eluted proteins were analyzed using SDS-PAGE and Western blot analysis.

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ICAT transfection

HUVECs (Cambrex, Walkersville, MD) were grown and maintained in endothelial growth medium-2 (EGM-2; Cambrex). Cells were transfected with plasmid pFLAG-CMV2/ICAT (14) or empty vector (mock) with the Nucleofector II Device (Amaxa Biosystems, Cologne, Germany) according to the manufacturer's instructions. Briefly, HUVECs grown to 80–90% confluence in EGM-2 were trypsinized and washed with PBS. The number of cells was counted, the suspension was centrifuged at 100 *g* for 10 min, and the pellet was resuspended in HUVEC nucleofector solution (Amaxa Biosystems) at 5×10^6 cells/ml. Plasmid DNA (2 μg) was added to 100 μl of the cell suspension, and the mixture was transferred into a cuvette for nucleofection. Immediately after nucleofection, 500 μl of prewarmed EGM-2 were added to the cuvette, and, after a 15-min incubation at 37°C, cells were seeded into either 35- or 60-mm culture dishes. At 4–6 h posttransfection, cells were washed with PBS, and dishes were refilled with fresh medium. Cells were used for study at 2–3 days posttransfection.

Transendothelial electrical resistance

The endothelial barrier property related to cell-cell adhesions was evaluated by measuring transendothelial electrical resistance (TER) as we previously described (5). Briefly, HUVECs were transfected with pFLAG/ICAT or empty vector and, at 48 h posttransfection, were subcultured onto ECIS electrode arrays (Applied Biophysics, Troy, NY) at 10^5 cell/cm^2 and grown overnight at 37°C. With culture medium serving as the electrode, barrier function was dynamically measured by determining the electrical impedance of a cell-covered electrode. A 1-V, 4,000-Hz alternating current signal was supplied through a 1-MΩ resistor to approximate a constant-current source. The in-phase voltage (proportional to resistance) and out-of-phase voltage (proportional to capacitive resistance) were measured and analyzed with ECMS 1.0 software (CET, Coralville, IA). Endothelial barrier function was expressed as TER normalized to baseline. Only endothelial monolayers with a baseline TER of 5,000 Ω or higher were used for experiments in this study.

Endothelial monolayer permeability to albumin

The transendothelial flux of albumin across cultured endothelial cell monolayers was measured using our previously described method (18, 39). Briefly, HUVECs were seeded at a density of 3×10^5 cells/cm^2 on gelatin-coated Costar Transwell membranes (Corning, Corning, NY) and grown 2–3 days until confluent. Cells were washed with endothelial basal medium (EBM) supplemented with 0.5% FBS and incubated with the same medium for 1 h. Histamine (100 μM) or an equivalent volume of vehicle was added to the top (luminal) chamber, followed by the addition of FITC-labeled BSA (Sigma) at a concentration of 1.0 mg/ml. Plates were incubated at 37°C for 30 min, and samples were removed from both the top and bottom (abluminal) chambers for fluorometry analysis. The readings were converted to albumin concentrations with the use of a standard curve. The sample concentrations were then used in the following equation to determine the apparent permeability coefficient of albumin (P_a): $P_a = [A]/t \times 1/A \times V/[L]$, where [A] is the abluminal concentration, t is time (in s), A is the area of the membrane (in cm^2), V is the volume of the abluminal chamber, and [L] is the luminal concentration.

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Immunoprecipitation and Western blot analysis

HUVECs were grown and maintained routinely in gelatin-coated dishes containing EGM-2. Medium was changed to EBM (Cambrex) supplemented with 0.5% FBS for 4 h before the collection of lysates. Cells were lysed in a Tris·HCl lysis buffer containing 1% Triton X-100 and protease inhibitors. The lysate was clarified by centrifugation at 13,000 *g* for 20 min, and supernatants were incubated for 4 h with various antibodies preadsorbed onto protein A/G-coupled agarose beads (Santa Cruz Biotechnology) for pull down of respective proteins. Proteins eluted from beads or in total lysates were separated by SDS-PAGE, transferred to nitrocellulose or polyvinylidene difluoride membranes, and blotted with monoclonal antibodies to β-catenin, VE-cadherin, or the His tag. The membrane was incubated with the corresponding horseradish peroxidase-conjugated secondary antibody, and immunoreactive bands were detected using the Pico Supersignal chemiluminescent substrate (Pierce). Images of the blots were acquired by reflectance scanning densitometry, and band intensity was quantified using National Institutes of Health Image software.

Immunofluorescence microscopy

HUVECs grown on gelatin-coated coverslips were fixed with 3% paraformadehyde and permeabilized with 0.2% Triton X-100. For the labeling of recombinant ICAT, cells were incubated with a monoclonal anti-FLAG antibody (Sigma) for 1.5 h, followed by an incubation with FITC-labeled anti-mouse IgG (Santa Cruz Biotechnology). Coverslips were then mounted onto slides for fluorescence microscopy and viewed with an Axiovert 200M fluorescent microscope equipped with an AxioCam MRm camera (Carl Zeiss, Thornwood, NY). Digital images were collected with Zeiss Axiovision 4.0 software.

Data analyses

For all experiments, n is given as the number of dishes or wells of endothelial cells studied. Data are expressed as means ± SE. Statistical analysis with ANOVA followed by Bonferroni t-tests was performed to evaluate the significance of intergroup differences. Significance was accepted at $P < 0.05$.

Results

ICAT expression and binding interactions with β-catenin in vitro

The molecular structure of ICAT and its interaction with β-catenin are shown in Fig. 1. β-Catenin uses the same armadillo (arm) repeat domain to bind to several different intracellular ligands, including ICAT (Fig. 1A). Sequence alignment revealed that, like other members of the cadherin family, VE-cadherin shares with ICAT the conserved and critical amino acid residues involved in binding interactions with β-catenin (Fig. 1B), and ICAT binds to β-catenin arm repeats 5–12 in a manner similar to that of cadherins (10, 15). To demonstrate that ICAT binds β-catenin, recombinant ICAT was expressed and purified as a 9.6-kDa NH2-terminal, His-tagged fusion protein. This recombinant protein reacted with both anti-His monoclonal antibody and polyclonal antiserum against ICAT (Fig. 1C). Recombinant ICAT was able to bind to β-catenin from endothelial cells in a concentration-related pattern (Fig. 1D). Under the same conditions, blank Ni-NTA agarose beads did not

show the adsorption of detectable β-catenin, corroborating the specificity of His-ICAT and β-catenin binding interactions. Importantly, by using in vitro competition binding assay (Fig. 2) in which GST-β-catenin-coupled glutathione agarose beads were incubated with the recombinant VE-cadherin CPD in the presence of different amounts of His-tagged ICAT, we found that the level of VE-cadherin bound to β-catenin beads decreased as a function of increased concentration of ICAT, confirming that ICAT effectively inhibited the binding of (3-catenin to VE-cadherin in a concentration-related manner (Fig. 2).

Endothelial junction protein expression during ICAT over-expression

To determine the potential impact of ICAT on endothelial cell-cell adhesions, FLAG-ICAT protein was over-expressed in HUVECs. As shown in Fig. 3A, transfection of pFLAG/ICAT with the aid of Nucleofector II yielded a high transfection efficiency in HUVECs without damaging cell viability and function. Transfection at 2 μg produced an optimal, high level of ICAT expression; thus, this concentration was used in subsequent experiments. Further analyses in fractionated cell lysates revealed a preferential distribution of the transfected ICAT in the cytosolic pool, with relatively less amount of protein detected in the nuclear extract and essentially none in the cell membrane (Fig. 3B), confirming the intracellular location of ICAT, consistent with a previous finding with epithelial cells. Interestingly, overexpression of recombinant ICAT did not significantly alter expression levels of VE-cadherin and β-catenin in endothelial cells (Fig. 3C).

Endothelial barrier function during ICAT overexpression

To determine whether ICAT expression affects endothelial barrier function, we first assessed the TER of HUVECs transfected with ICAT. During the attachment of cells to the electrodes and subsequent monolayer formation, we observed no differences in the basal TER between FLAG-ICAT-transfected and mock-transfected HUVECs (data not shown). On the other hand, cells overexpressing ICAT displayed a greater response to histamine (Fig. 4), as both the magnitude (Fig. 4B) and duration (Fig. 4C) of the decrease in TER were significantly augmented in pFLAG-ICAT-transfected cells compared with nontransfected and mock-transfected cells, indicating that ICAT delays the recovery of histamine-induced barrier dysfunction (see Supplemental Table 1 for electrical resistance values).[1]

We then further examined the ICAT effect on endothelial permeability to protein. We found that the overexpression of ICAT did not affect basal P_{a}, as there were no significant differences in P_a between ICAT-transfected and mock-transfected cells. In contrast, HUVECs overexpressing ICAT displayed a significantly greater hyperpermeability response to histamine than mock-transfected cells (Fig. 5), consistent with the augmented decrease in electrical resistance (Fig. 4).

β-Catenin/VE-cadherin binding during ICAT overexpression

Immunoprecipitation and Western blot analysis revealed no difference in β-catenin-bound VE-cadherin levels between mock- and ICAT-transfected endothelial cells under the basal, nonstimulated conditions (Fig. 6). After 2–5 min of histamine treatment, however, both

[1]Supplemental data for this article is available online at the *American Journal of Physiology-Cell Physiology* website.

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ICAT- and mock-transfected cells showed significant decreases in the pool of β-catenin-bound VE-cadherin, indicating dissociation of the two molecules. The response, although not statistically significant, appeared to be more severe in cells overexpressing ICAT. Interestingly, at 10 min after histamine treatment, there was a sustained decrease in the level of β-catenin-associated VE-cadherin in ICAT-expressing cells, whereas mock-transfected cells showed reassociation of β-catenin and VE-cadherin. At later time points (30 or 60 min) of treatment, the fractions of β-catenin-associated VE-cadherin in both groups of cells returned to pretreatment levels (Fig. 6).

Discussion

In this study, we present evidence suggesting a dynamic response of the endothelial adherens junction to acute inflammatory stimulation, in that dissociation of the VE-cadherin/β-catenin complex augments the extent, and delays the recovery, of histamine-induced hyperpermeability. This notion is supported by data showing that overexpression of ICAT, which competed with VE-cadherin to bind β-catenin, did not affect basal barrier function but increased the magnitude as well as the duration of the hyperpermeability response to histamine in endothelial cells. Moreover, we found that histamine caused a time-dependent dissociation of β-catenin from VE-cadherin concomitant with elevated permeability, which was enhanced with the overexpression of ICAT. Combined with our previous finding that experimental disruption of the adherens junction elevates microvascular permeability (18, 39), these results suggest that certain inflammatory stimuli may increase endothelial permeability through a mechanism involving the dissociation of β-catenin and VE-cadherin from the adherens junction. We have not yet tested whether the ICAT-induced exacerbation of barrier dysfunction is universal or histamine specific. We speculate that ICAT may affect the endothelial permeability response to other agonists in which the mechanism predominantly involves changes in adherens junctions.

Endothelial junctions are dynamically regulated by physical forces, cellular factors, and chemical mediators (17, 24). We and others have previously demonstrated that inflammatory mediators, including histamine, thrombin, growth factors, cytokines, oxidants, and activated neutrophils, can increase the transendothelial flux of fluids, macromolecules, and circulating cells across the microvascular wall, mainly via a paracellular route (3, 11, 23, 25, 27, 34, 49). Alterations in endothelial junction morphology and intercellular gap formation are typically observed concomitantly with fluid or macromolecule leakage. The homotypic binding of VE-cadherin molecules on adjacent endothelial cells is critical for the maintenance of intercellular junctions, and interfering with this binding by depleting extracellular calcium or introducing VE-cadherin monoclonal antibodies disrupts the junctions and elevates paracellular permeability (1, 7, 8, 12). While such dramatic perturbation of the junctional structures may not reflect the pathophysiology of endothelial cells in inflammation, alternative mechanisms involving a more subtle and dynamic response in the junction have begun to be appreciated. Within this context, the CPD of VE-cadherin represents an important target for dynamic modification by the intracellular signaling cascades triggered upon inflammatory stimulation. We have previously shown the importance of the VE-cadherin CPD in maintaining the structural integrity of junctional complexes, as interrupting the normal binding between VE-cadherin and the cytoskeleton by

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the introduction of an ectopic VE-cadherin CPD fusion protein caused increased permeability in endothelial cell monolayers as well as in intact microvessels (18). The results from the present study build upon the concept that the dynamics of VE-cadherin and β-catenin binding interactions regulate the junctional integrity and thus barrier function in endothelial cells, suggesting that VE-cadherin/β-catenin dissociation and reassociation, respectively, serve as a mechanism for the initial increase in endothelial permeability and subsequent restoration of normal barrier function after stimulation by acute inflammatory mediators.

An interesting aspect of our results lies in the finding that ICAT is able to compete with VE-cadherin for the β-catenin binding site in vascular endothelial cells. ICAT is a newly identified protein capable of blocking the binding between C-cadherin or E-cadherin and β-catenin in epithelial cells (14, 15). Crystal structure analysis indicates that the ICAT 3-helix bundle binds to arm repeats 10–12 of β-catenin and that the COOH-terminal extended domain of ICAT overlaps with, and essentially obstructs, arm repeats 5–9, which contain the binding regions of T cell factor, adenomatous polyposis coli (APC), and E-cadherin (10, 15). The sequence of the VE-cadherin CPD shares several highly conserved residues at critical positions in the β-catenin binding region (corresponding to the COOH-terminal tail of ICAT), and our in vitro binding assay confirmed that ICAT can compete with VE-cadherin for β-catenin binding in a concentration-dependent manner. Thus, in this study, we used ICAT primarily to inhibit the β-catenin binding to VE-cadherin. It is worth noting that this treatment may also cause inhibition of β-catenin's ability to activate T cell factor/lymphocyte enhancer factor (LEF). In endothelial cells, T cell factor/LEF activation promotes the expression of genes associated with angiogenesis (26). Therefore, prolonged overexpression of ICAT may inhibit endothelial cell proliferation or migration. Also, we did not examine whether ICAT altered VE-cadherin binding to other catenins, such as plakoglobin, which have been shown to be key elements in the assembly of intercellular junctions and thus modulate barrier function (32, 40). We expect that interference with plakoglobin binding to VE-cadherin would promote a similar effect as disruption of VE-cadherin/β-catenin binding.

Although the present observations were limited to a relatively short term, overexpression of ICAT may exert permeability effects over longer periods of time in the absence of inflammatory stimuli. This possibility is supported by the finding that adherens junctions are constantly undergoing remodeling with VE-cadherin turnover (40). However, it is unlikely that ICAT affects the de novo formation of adherens junctions in the same way as reannealing of disrupted junctions following an inflammatory stimulus, as we did not detect any apparent change in VE-cadherin and β-catenin levels in ICAT-overexpressing cells, and these cells produced electrical resistance and solute permeability comparable with those of mock-treated cells in the absence of inflammatory stimulation. Thus, ICAT may not significantly affect VE-cadherin turnover or disrupt junctional integrity in the absence of inflammatory stimulation. This was not surprising, considering that an injection of ICAT mRNA or protein into *Xenopus* embryos did not disrupt cell-cell adhesion or block the binding of C-cadherin to β-catenin during development (15) and that Madin-Darby canine kidney (MDCK) epithelial cells stably expressing epitope-tagged ICAT do not show evident changes in cadherin-based cell adhesion (14). On the other hand, posttranslational

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modifications of cadherins could potentially alter the ability of ICAT to competitively bind to β-catenin. For example, phosphorylation of serine residues in the common sequence motif SLSSL in both E-cadherin and APC tremendously increases their binding affinity for β-catenin (6, 21). Moreover, both E-cadherin and VE-cadherin form tight complexes with β-catenin once they appear in the endoplasmic reticulum, and these complexes move as individual units to the surface of respective cells for establishing cell adhesion (28). Additional factors to consider are how the formation of multiprotein complexes containing VE-cadherin and β-catenin affects their binding to each other as well as localization at specific subcellular compartments.

Although we saw no noticeable changes in cells overexpressing ICAT under basal conditions, after histamine stimulation these cells displayed an enhanced and prolonged hyperpermeability response in association with VE-cadherin/β-catenin complex dissociation. This pattern was also previously observed in MDCK cells expressing ICAT, with no changes during nonstimulated conditions but an enhanced cell scattering upon stimulation with hepatocyte growth factor, suggesting the involvement of ICAT in dynamic rather than steady-state cell adhesions (14). Histamine, upon binding to its receptors on endothelial cells, triggers several signal transduction cascades including activation of PLC and PKC, intracellular calcium mobilization, nitric oxide production, activation of MAPKs, myosin light chain phosphorylation and actin-myosin contraction, and tyrosine phosphorylation of junctional proteins (3, 4, 20, 29, 43, 50); all of these reactions contribute to the hyperpermeability response. Of particular note, tyrosine phosphorylation of β-catenin decreases its affinity for cadherins, resulting in the dissociation of β-catenin from the cadherin complex and weakened cell-cell adhesions (19, 28, 31). Based on these findings, we speculate that histamine promotes conditions that allow ICAT to capture β-catenin dissociated from VE-cadherin, slowing the reassembly of β-catenin/VE-cadherin complexes and thus the recovery of hyperpermeability.

Further analyses of the pattern of electrical barrier responses shown in Fig. 4 may provide some mechanistic insight regarding histamine-induced hyperpermeability. Clearly, ICAT over-expression enhanced the initial drop of TER upon histamine treatment, indicative of exacerbated barrier dysfunction. Considering that the recovery process in ICAT-expressing cells started at a lower set point, one may reason that the slower recovery might be due to the initial drop in TER. In other words, the rate of recovery may decrease as a function of the magnitude of loss of barrier function. While this is plausible, the Western blot data (Fig. 6) showed a delayed reassociation of VE-cadherin with β-catenin in a time course correlating with that of the TER response in ICAT-expressing cells, indicating an effect of prolonged VE-cadherin/β-catenin dissociation to cause delayed barrier recovery. On the other hand, an interesting phenomenon seen in mock-transfected cells is that, following the initial drop in TER after histamine, the barrier function not only quickly returned to the basal level but further increased (Fig. 4), which appeared to occur without an associated increase in VE-cadherin/β-catenin binding (Fig. 6). This suggests that different mechanisms may be involved in the barrier restoration process after inflammatory stimulation. One explanation would be that adherens junction dissociation triggers multiple recovery signaling events leading to an enhanced barrier defense. Another possibility is that other intercellular adhesion molecules, such as the tight junction, contribute to the recovery

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process where reassociation of VE-cadherin and β-catenin may further signal the reorganization of these cell-cell adhesive structures.

In summary, we show that histamine alters the binding interaction between VE-cadherin and β-catenin in association with hyperpermeability in endothelial cells. Overexpression of ICAT enhances and prolongs histamine-induced VE-cadherin-β-catenin complex dissociation and the hyperpermeability response. These results suggest that VE-cadherin and β-catenin binding dynamics are important determinants in microvascular barrier regulation.

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References

1. Alexander JS, Alexander BC, Eppihimer LA, Goodyear N, Haque R, Davis CP, Kalogeris TJ, Carden DL, Zhu YN, Kevil CG. Inflammatory mediators induce sequestration of VE-cadherin in cultured human endothelial cells. Inflammation. 2000; 24:99–113. [PubMed: 10718113]
2. Allport JR, Ding H, Collins T, Gerritsen ME, Luscinskas FW. Endothelial-dependent mechanisms regulate leukocyte transmigration: a process involving the proteasome and disruption of the vascular endothelial-cadherin complex at endothelial cell-to-cell junctions. J Exp Med. 1997; 186:517–527. [PubMed: 9254650]
3. Andriopoulou P, Navarro P, Zanetti A, Lampugnani MG, Dejana E. Histamine induces tyrosine phosphorylation of endothelial cell-to-cell adherens junctions. Arterioscler Thromb Vasc Biol. 1999; 19:2286–2297. [PubMed: 10521356]
4. Baldwin AL, Thurston G. Changes in endothelial actin cytoskeleton in venules with time after histamine treatment. Am J Physiol Heart Circ Physiol. 1995; 269:H1528–H1537.
5. Breslin JW, Sun H, Xu W, Rodarte C, Moy AB, Wu MH, Yuan SY. Involvement of ROCK-mediated endothelial tension development in neutrophil-stimulated microvascular leakage. Am J Physiol Heart Circ Physiol. 2006; 290:H741–H750. [PubMed: 16172166]
6. Choi HJ, Huber AH, Weis WI. Thermodynamics of beta-catenin-ligand interactions: the roles of the N- and C-terminal tails in modulating binding affinity. J Biol Chem. 2006; 281:1027–1038. [PubMed: 16293619]
7. Corada M, Liao F, Lindgren M, Lampugnani MG, Breviario F, Frank R, Muller WA, Hicklin DJ, Bohlen P, Dejana E. Monoclonal antibodies directed to different regions of vascular endothelial cadherin extracellular domain affect adhesion and clustering of the protein and modulate endothelial permeability. Blood. 2001; 97:1679–1684. [PubMed: 11238107]
8. Corada M, Mariotti M, Thurston G, Smith K, Kunkel R, Brockhaus M, Lampugnani MG, Martin-Padura I, Stoppacciaro A, Ruco L, McDonald DM, Ward PA, Dejana E. Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. Proc Natl Acad Sci USA. 1999; 96:9815–9820. [PubMed: 10449777]
9. Cullere X, Shaw SK, Andersson L, Hirahashi J, Luscinskas FW, Mayadas TN. Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. Blood. 2005; 105:1950–1955. [PubMed: 15374886]
10. Daniels DL, Weis WI. ICAT inhibits beta-catenin binding to Tcf/Lef-family transcription factors and the general coactivator p300 using independent structural modules. Mol Cell. 2002; 10:573–584. [PubMed: 12408825]
11. Del Maschio A, Zanetti A, Corada M, Rival Y, Ruco L, Lampugnani MG, Dejana E. Polymorphonuclear leukocyte adhesion triggers the disorganization of endothelial cell-to-cell adherens junctions. J Cell Biol. 1996; 135:497–510. [PubMed: 8896605]

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12. Gao X, Kouklis P, Xu N, Minshall RD, Sandoval R, Vogel SM, Malik AB. Reversibility of increased microvessel permeability in response to VE-cadherin disassembly. Am J Physiol Lung Cell Mol Physiol. 2000; 279:L1218–L1225. [PubMed: 11076812]
13. Gottardi CJ, Gumbiner BM. Distinct molecular forms of beta-catenin are targeted to adhesive or transcriptional complexes. J Cell Biol. 2004; 167:339–349. [PubMed: 15492040]
14. Gottardi CJ, Gumbiner BM. Role for ICAT in β-catenin-dependent nuclear signaling and cadherin functions. Am J Physiol Cell Physiol. 2004; 286:C747–C756. [PubMed: 14613891]
15. Graham TA, Clements WK, Kimelman D, Xu W. The crystal structure of the beta-catenin/ICAT complex reveals the inhibitory mechanism of ICAT. Mol Cell. 2002; 10:563–571. [PubMed: 12408824]
16. Graham TA, Weaver C, Mao F, Kimelman D, Xu W. Crystal structure of a beta-catenin/Tcf complex. Cell. 2000; 103:885–896. [PubMed: 11136974]
17. Gumbiner BM. Regulation of cadherin adhesive activity. J Cell Biol. 2000; 148:399–404. [PubMed: 10662767]
18. Guo M, Wu MH, Granger HJ, Yuan SY. Transference of recombinant VE-cadherin cytoplasmic domain alters endothelial junctional integrity and porcine microvascular permeability. J Physiol. 2004; 554:78–88. [PubMed: 14678493]
19. Guo X, Rao JN, Liu L, Rizvi M, Turner DJ, Wang JY. Polyamines regulate β-catenin tyrosine phosphorylation via Ca^{2+} during intestinal epithelial cell migration. Am J Physiol Cell Physiol. 2002; 283:C722–C734. [PubMed: 12176729]
20. Huang Q, Yuan Y. Interaction of PKC and NOS in signal transduction of microvascular hyperpermeability. Am J Physiol Heart Circ Physiol. 1997; 273:H2442–H2451.
21. Huber AH, Weis WI. The structure of the beta-catenin/E-cadherin complex and the molecular basis of diverse ligand recognition by beta-catenin. Cell. 2001; 105:391–402. [PubMed: 11348595]
22. Iyer S, Ferreri DM, DeCocco NC, Minnear FL, Vincent PA. VE-cadherin-p120 interaction is required for maintenance of endothelial barrier function. Am J Physiol Lung Cell Mol Physiol. 2004; 286:L1143–L1153. [PubMed: 14672921]
23. Kevil CG, Payne DK, Mire E, Alexander JS. Vascular permeability factor/vascular endothelial cell growth factor-mediated permeability occurs through disorganization of endothelial junctional proteins. J Biol Chem. 1998; 273:15099–15103. [PubMed: 9614120]
24. Kusumi A, Suzuki K, Koyasako K. Mobility and cytoskeletal interactions of cell adhesion receptors. Curr Opin Cell Biol. 1999; 11:582–590. [PubMed: 10508652]
25. Leach L, Eaton BM, Westcott ED, Firth JA. Effect of histamine on endothelial permeability and structure and adhesion molecules of the paracellular junctions of perfused human placental microvessels. Microvasc Res. 1995; 50:323–337. [PubMed: 8583948]
26. Masckauchan TN, Shawber CJ, Funahashi Y, Li CM, Kitajewski J. Wnt/beta-catenin signaling induces proliferation, survival and interleukin-8 in human endothelial cells. Angiogenesis. 2005; 8:43–51. [PubMed: 16132617]
27. McDonald DM, Thurston G, Baluk P. Endothelial gaps as sites for plasma leakage in inflammation. Microcirculation. 1999; 6:7–22. [PubMed: 10100186]
28. Mehta D, Malik AB. Signaling mechanisms regulating endothelial permeability. Physiol Rev. 2006; 86:279–367. [PubMed: 16371600]
29. Moy AB, Winter M, Kamath A, Blackwell K, Reyes G, Giaever I, Keese C, Shasby DM. Histamine alters endothelial barrier function at cell-cell and cell-matrix sites. Am J Physiol Lung Cell Mol Physiol. 2000; 278:L888–L898. [PubMed: 10781418]
30. Potter MD, Barbero S, Cheresh DA. Tyrosine phosphorylation of VE-cadherin prevents binding of p120- and beta-catenin and maintains the cellular mesenchymal state. J Biol Chem. 2005; 280:31906–31912. [PubMed: 16027153]
31. Roura S, Miravet S, Piedra J, Garcia de Herreros A, Dunach M. Regulation of E-cadherin/catenin association by tyrosine phosphorylation. J Biol Chem. 1999; 274:36734–36740. [PubMed: 10593980]
32. Schnittler HJ, Puschel B, Drenckhahn D. Role of cadherins and plakoglobin in interendothelial adhesion under resting conditions and shear stress. Am J Physiol Heart Circ Physiol. 1997; 273:H2396–H2405.

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33. Shasby DM, Ries DR, Shasby SS, Winter MC. Histamine stimulates phosphorylation of adherens junction proteins and alters their link to vimentin. Am J Physiol Lung Cell Mol Physiol. 2002; 282:L1330–L1338. [PubMed: 12003790]
34. Siflinger-Birnboim A, Malik AB. Regulation of endothelial permeability by second messengers. New Horiz. 1996; 4:87–98. [PubMed: 8689278]
35. Stevens T, Garcia JG, Shasby DM, Bhattacharya J, Malik AB. Mechanisms regulating endothelial cell barrier function. Am J Physiol Lung Cell Mol Physiol. 2000; 279:L419–L422. [PubMed: 10956614]
36. Tago K, Nakamura T, Nishita M, Hyodo J, Nagai S, Murata Y, Adachi S, Ohwada S, Morishita Y, Shibuya H, Akiyama T. Inhibition of Wnt signaling by ICAT, a novel beta-catenin-interacting protein. Genes Dev. 2000; 14:1741–1749. [PubMed: 10898789]
37. Tinsley JH, Breslin JW, Teasdale NR, Yuan SY. PKC-dependent, burn-induced adherens junction reorganization and barrier dysfunction in pulmonary microvascular endothelial cells. Am J Physiol Lung Cell Mol Physiol. 2005; 289:L217–L223. [PubMed: 15821015]
38. Tinsley JH, Ustinova EE, Xu W, Yuan SY. Src-dependent, neutrophil-mediated vascular hyperpermeability and β-catenin modification. Am J Physiol Cell Physiol. 2002; 283:C1745–C1751. [PubMed: 12388068]
39. Tinsley JH, Wu MH, Ma W, Taulman AC, Yuan SY. Activated neutrophils induce hyperpermeability and phosphorylation of adherens junction proteins in coronary venular endothelial cells. J Biol Chem. 1999; 274:24930–24934. [PubMed: 10455168]
40. Vincent PA, Xiao K, Buckley KM, Kowalczyk AP. VE-cadherin: adhesion at arm's length. Am J Physiol Cell Physiol. 2004; 286:C987–C997. [PubMed: 15075197]
41. Wang Y, Lewis DF, Gu Y, Zhang Y, Alexander JS, Granger DN. Placental trophoblast-derived factors diminish endothelial barrier function. J Clin Endocrinol Metab. 2004; 89:2421–2428. [PubMed: 15126573]
42. Wong RK, Baldwin AL, Heimark RL. Cadherin-5 redistribution at sites of TNF-α and IFN-γ-induced permeability in mesenteric venules. Am J Physiol Heart Circ Physiol. 1999; 276:H736–H748.
43. Wu MH, Yuan SY, Granger HJ. The protein kinase MEK1/2 mediate vascular endothelial growth factor- and histamine-induced hyperpermeability in porcine coronary venules. J Physiol. 2005; 563:95–104. [PubMed: 15539400]
44. Xu M, Waters CL, Hu C, Wysolmerski RB, Vincent PA, Minnear FL. Sphingosine 1-phosphate rapidly increases endothelial barrier function independently of VE-cadherin but requires cell spreading and Rho kinase. Am J Physiol Cell Physiol. 2007; 293:C1309–C1318. [PubMed: 17670896]
45. Yap AS, Niessen CM, Gumbiner BM. The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. J Cell Biol. 1998; 141:779–789. [PubMed: 9566976]
46. Yuan SY. Signal transduction pathways in enhanced microvascular permeability. Microcirculation. 2000; 7:395–403. [PubMed: 11142336]
47. Yuan SY, Breslin JW, Perrin R, Gaudreault N, Guo M, Kargozaran H, Wu MH. Microvascular permeability in diabetes and insulin resistance. Microcirculation. 2007; 14:363–373. [PubMed: 17613808]
48. Yuan SY, Ustinova EE, Wu MH, Tinsley JH, Xu W, Korompai FL, Taulman AC. Protein kinase C activation contributes to microvascular barrier dysfunction in the heart at early stages of diabetes. Circ Res. 2000; 87:412–417. [PubMed: 10969040]
49. Yuan SY, Wu MH, Ustinova EE, Guo M, Tinsley JH, De Lanerolle P, Xu W. Myosin light chain phosphorylation in neutrophil-stimulated coronary microvascular leakage. Circ Res. 2002; 90:1214–1221. [PubMed: 12065325]
50. Yuan Y, Granger HJ, Zawieja DC, DeFily DV, Chilian WM. Histamine increases venular permeability via a phospholipase C-NO synthaseguanylate cyclase cascade. Am J Physiol Heart Circ Physiol. 1993; 264:H1734–H1739.

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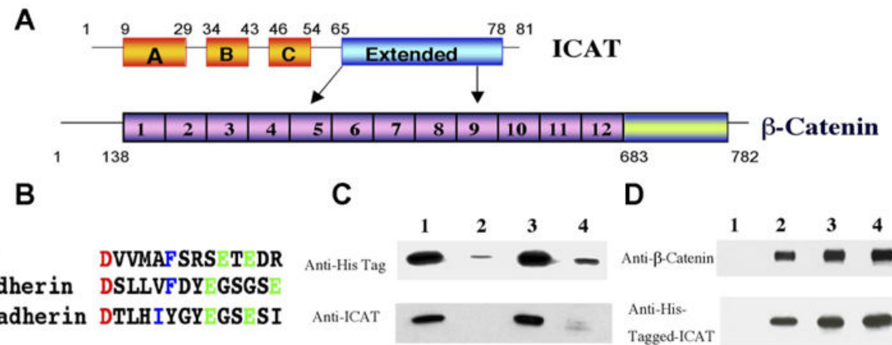

Fig.1.

Expression, purification, and analysis of recombinant human inhibitor of β-catenin and T cell factor (ICAT). *A:* schematics of the ICAT molecular structure and β-catenin binding motif. *Regions A*, *B*, and *C* represent three helixes of the NH_2-terminal, which binds to armadillo (arm) repeats 10–12, whereas the COOH-terminal extended region binds to arm repeats 5–9 of β-catenin. *B:* sequence alignment and comparison of the ICAT extended domain with the β-catenin binding regions of E-cadherin and VE-cadherin. [Adapted from Ref. 15] *C:* Western blots (WBs) for the detection of recombinant ICAT in purified preparations and *Esherichia coli* lysates using monoclonal anti-His antibody or rabbit polycolonal antibody to ICAT. *Lane 1*, purified protein; *lane 2*, mock extract from *E. coli* cells without isopropyl-β-D-thioglactoside (IPTG) induction; *lanes 3* and *4*, lysates with and without IPTG. *D:* in vitro binding interactions of His-ICAT and β-catenin from endothelial cells. His-tagged ICAT was immobilized on nickel-nitrioltriacetic acid agarose beads. Equal amounts of human umbilical vein endothelial cell (HUVEC) lysate were added to different amounts of ICAT-bound beads, followed by an incubation at 4°C for 2 h. After a wash, proteins were eluted from the beads in sample buffer, separated by SDS-PAGE on 4–20% Tris-glycine gel, and analyzed by immunoblot analysis with anti-His and anti-β-catenin antibodies, respectively. *Lane 1*, blank beads 45 μl; *lane 2*, blank beads 30 μl + His-ICAT beads 15 μl; *lane 3*, blank beads 15 μl + His-ICAT beads 30 μl; *lane 4*, His-ICAT beads 45 μl. WBs are representative of 3–5 separate experiments.

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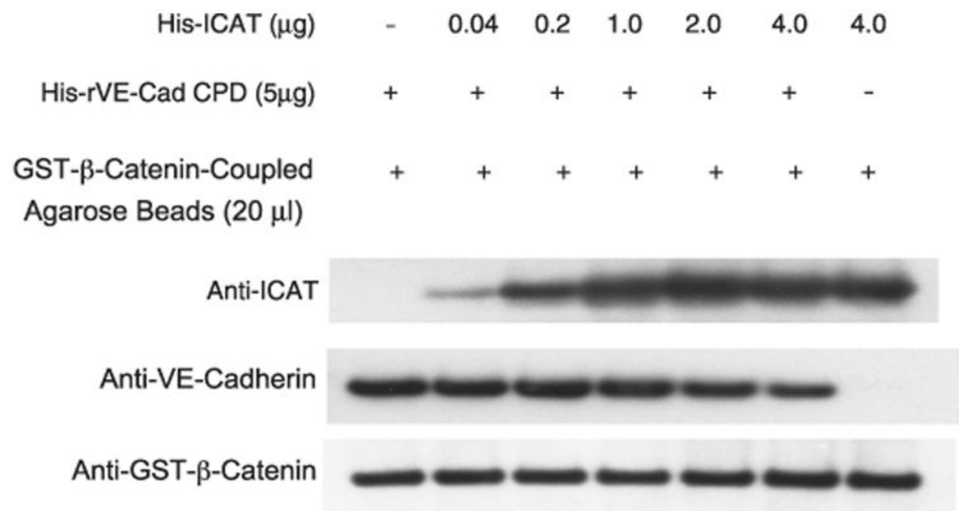

Fig.2.

ICAT competes VE-cadherin for β-catenin binding. The recombinant (r)VE-cadherin cytoplasmic domain (CPD) was incubated with GST-β-catenin (residues 134–664)-coupled glutathione agarose beads in the presence of different amounts of His-tagged ICAT. WB analysis was performed to quantify the amount of ICAT and VE-cadherin bound to the beads. The level of VE-cadherin bound to β-catenin beads decreased as a function of the increasing concentration of ICAT. WBs are representative of 3 separate experiments.

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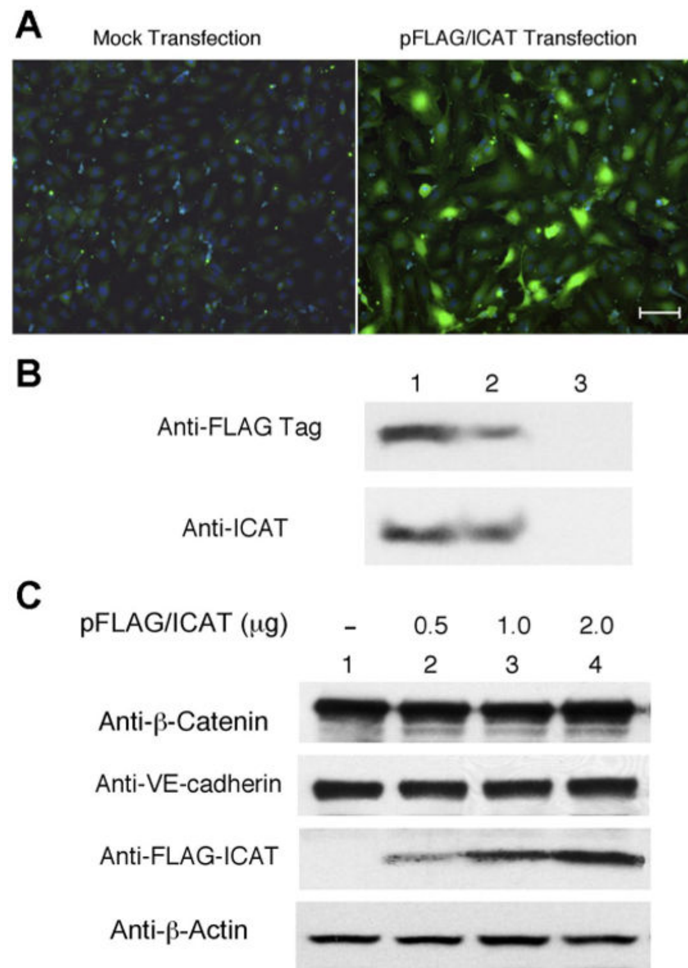

Fig.3.

Expression and distribution of FLAG-tagged ICAT in HUVECs and its effect on the endothelial expression of β-catenin and VE-cadherin. *A:* representative images of HUVECs transfected with pFLAG-CM2/ICAT (pFLAG/ICAT) or empty vector (mock) at 2 days posttransfection. Cells were fixed, permeabilized, and labeled with Hoechst 33342 for nuclei (blue) staining and anti-FLAG antibody (green). Bar = 100 μm. *B:* subcellular distribution of FLAG-tagged ICAT protein. HUVECs transfected with pFLAG/ICAT were lysed in HEPES hypotonic buffer and fractionated by centrifugation. Cytosolic and membranous fractions were separated by ultracentrifugation at 100,000 *g* for 1 h. Subcellular fractions were subjected to SDS-PAGE and WB analysis. *Lane 1*, cytosolic fraction; *lane 2*, crude nuclear extract; *lane 3*, cell membrane fraction. *C*: expression of VE-cadherin and β-catenin in HUVECs transfected with different concentrations of ICAT. Cells transfected with pFLAG/ICAT or empty vector (mock) were lysed in Tris lysis buffer. Equal amounts of proteins in cell lysates were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and blotted with antibodies to VE-cadherin, β-catenin, the FLAG tag, and actin (loading control), respectively. Lanes are as follows: cells transfected with pFLAG-CM2/ICAT at 0 (*lane 1*), 0.5 (*lane 2*), 1.0 (*lane 3*), and 2.0 μg (*lane 4*). WBs are representative of 3 separate experiments at 2 days posttransfection.

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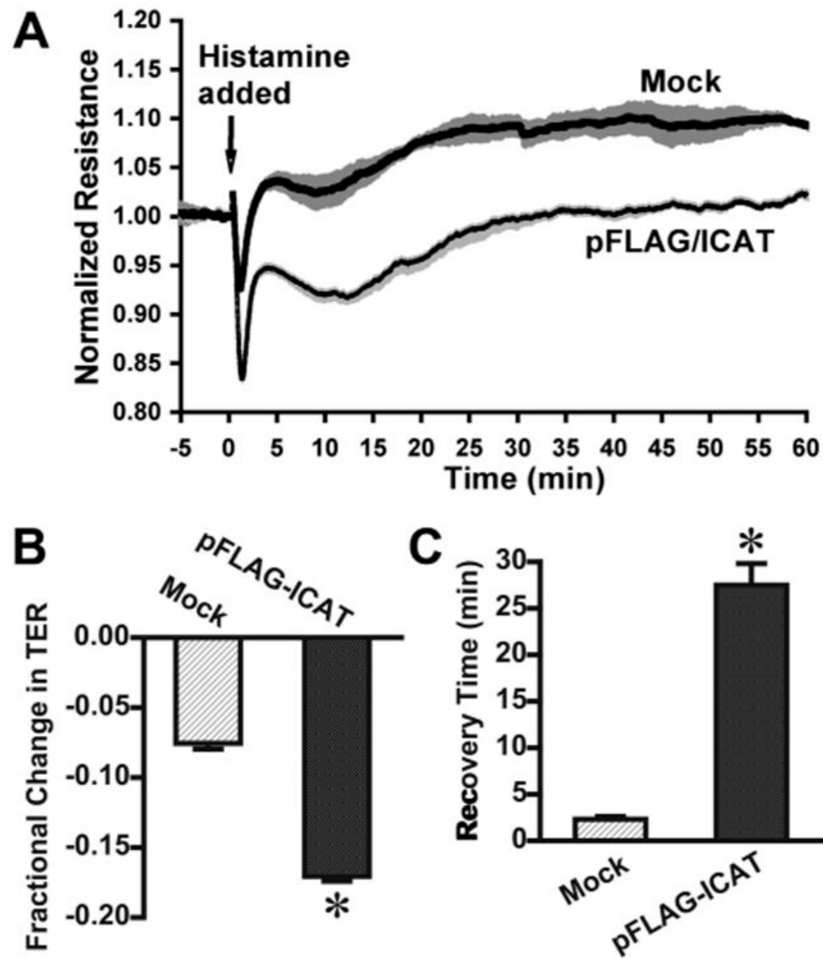

Fig.4.

ICAT augments histamine-induced endothelial barrier dysfunction as indicated by decreased transendothelial electric resistance (TER). *A:* HUVECs transfected with pFLAG/ICAT or empty vector (mock) were grown overnight in endothelial growth medium on electrode arrays of an electrical cell impedance sensor device. The medium was changed to endothelial basal medium with 0.5% FBS 6 h before treatment with histamine (0.1 mM). Tracings represent mean TERs for both groups, and the shaded areas represent SEs. *B:* the maximal change in TER, expressed as a fractional change from baseline, was significantly greater in HUVECs expressing FLAG-ICAT. *C*: the time for recovery to the basal TER value was significantly longer in FLAG-ICAT-expressing HUVECs. $^*P < 0.01$ vs. the mock transfection group. For the mock transfection group, $n = 4$; for the pFLAG/ICAT group, $n = 6$.

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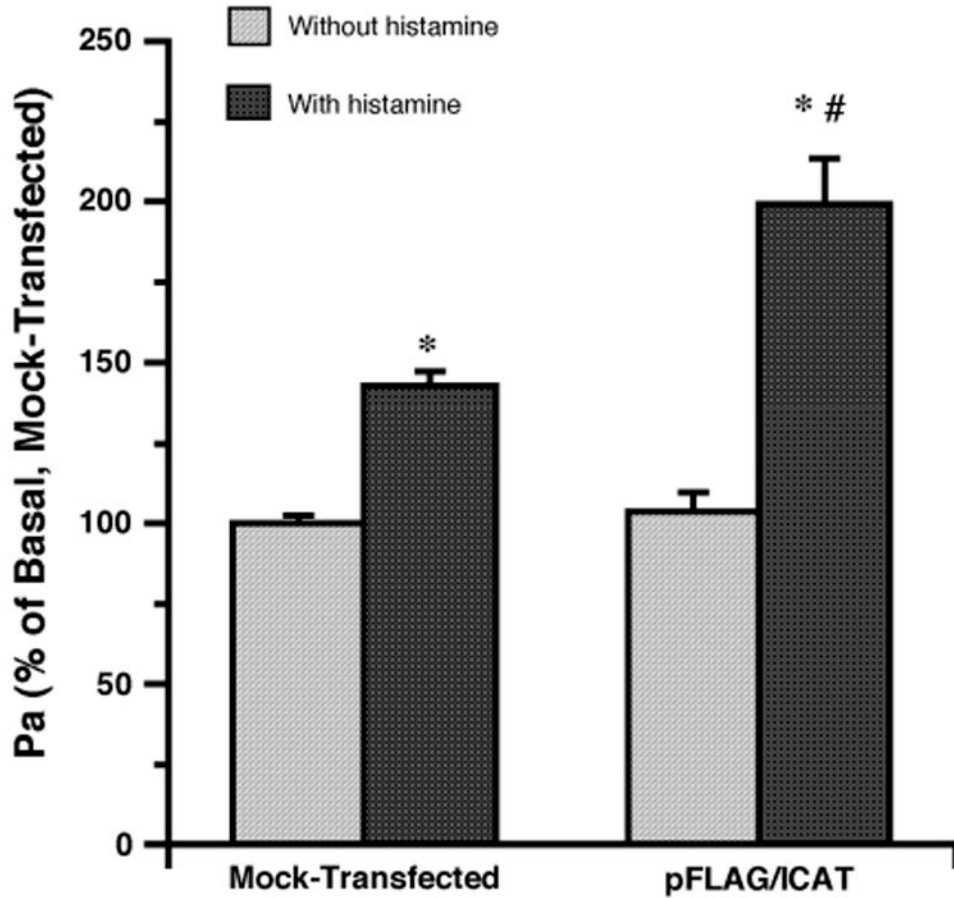

Fig. 5.

ICAT overexpression enhances histamine-induced endothelial barrier dysfunction as indicated by increased albumin flux across the endothelial monolayer. HUVECs were transfected with either pFLAG/ICAT or empty vector (mock). Cells were seeded onto gelatin-coated Costar Transwell membranes and grown to confluence. Cells were treated with histamine at 0.1 mM or vehicle, followed by the addition of FITC-albumin. The permeability of albumin (P_a) was calculated as described in materials and methods. P_a values are expressed as percentages of the basal level (of mock transfection in the absence of histamine). For each treatment, $n = 5$. *$P < 0.05$ vs. basal (mock transfection without histamine); #$P < 0.05$ vs. mock transfection with histamine stimulation.

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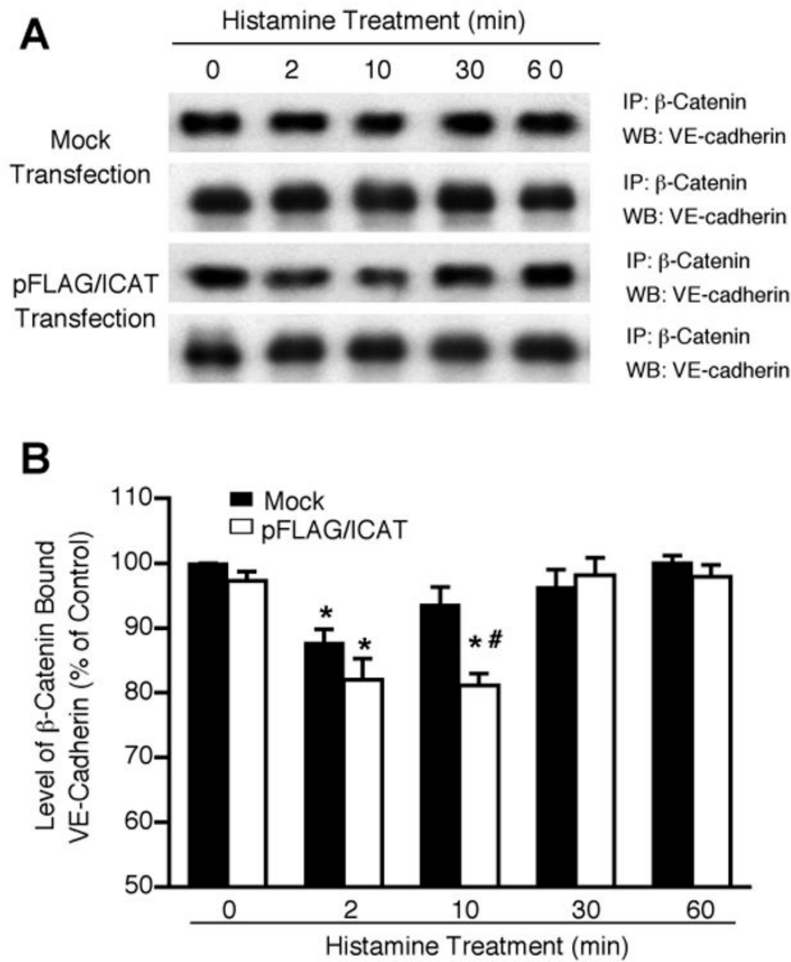

Fig. 6.

Effect of ICAT on histamine-induced VE-cadherin/β-catenin dissociation. HUVECs were transfected with either pFLAG/ICAT or empty vector (mock). Cells were treated with histamine (0.1 mM) or vehicle. After cell lysis, β-catenin was immunoprecipitated from individual cell lysates. Both β-catenin and VE-cadherin in the immunoprecipitates were analyzed by SDS-PAGE and WB analysis. *A:* WBs representative of 4 separate experiments. *B:* WBs were quantified using the NIH Image software, and levels of VE-cadherin associated with β-catenin were normalized to those of mock-transfected cells with no histamine treatment. IP, immunoprecipitation. Data are expressed as means ± SE; $n = 4$. *$P < 0.05$ vs. mock transfection without histamine; #$P < 0.05$ vs. mock transfection with histamine stimulation at the same points.