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Enhanced interaction between focal adhesion and adherens junction proteins: Involvement in sphingosine 1-phosphate-induced endothelial barrier enhancement

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Abstract

Sphingosine 1-phosphate (S1P) is an important vascular barrier regulatory agonist which enhances the junctional integrity of human lung endothelial cell monolayers. We have now demonstrated that S1P induced cortical actin ring formation and redistribution of focal adhesion kinase (FAK) and paxillin to the cell periphery suggesting the critical role of cell–cell adhesion in endothelial barrier enhancement. Co-immunoprecipitation studies revealed increased association of VE-cadherin with FAK and paxillin in S1P-challenged human pulmonary artery endothelial cell (HPAEC) monolayers. Furthermore, S1P-induced enhancement of VE-cadherin interaction with α -catenin and β -catenin was associated with the increased formation of FAK– β -catenin protein complexes. Depletion of β -catenin (siRNA) resulted in loss of S1P-mediated VE-cadherin association with FAK and paxillin rearrangement. Furthermore, transendothelial electrical resistance (an index of barrier function) demonstrated that β -catenin siRNA significantly attenuated S1P-induced barrier enhancement. These results demonstrate a mechanism of S1P-induced endothelial barrier enhancement via β -catenin-linked adherens junction and focal adhesion interaction.

Keywords

β -catenin; α -catenin; Sphingosine 1-phosphate; Focal adhesion; Adherens junction; Focal adhesion kinase; Paxillin; Endothelial; VE-cadherin; F-actin

Introduction

Sphingosine 1-phosphate (S1P) is a highly effective endothelial cell agonist that induces proliferation, calcium mobilization, adhesion molecule expression, and suppression of apoptosis (An et al., 2000; English et al., 2001; Goetzl and An, 1998; Hisano et al., 1999; Lee et al., 1999). S1P is present in human serum at micromolar levels (Yatomi et al., 1995; Murata et al., 2000; Panetti, 2002) and binds to the S1P family of receptors upon release by a variety of activated cells including endothelium and stimulated platelets (Lee et al., 1999,

Lee et al., 1998; Wang et al., 1999; Ishii et al., 2004). Our prior studies were the first to demonstrate that S1P is also an important endothelial barrier-regulatory agonist, which enhances vascular barrier integrity via ligation of the S1P receptor, S1P₁, and subsequent evoked signaling events (Garcia et al., 2001; McVerry and Garcia, 2004, 2005; Singleton et al., 2005; Dudek et al., 2004; Mehta and Malik, 2006; Komarova and Mehta, 2007).

Both focal adhesion (FA) and adherens junction (AJ) are critical to endothelial barrier regulation via FA proteins such as focal adhesion kinase (FAK) and paxillin as well as AJ proteins such as VE-adherin, α and β -catenins (Guo et al., 2004; Wu et al., 2003; Guo et al., 2005). We and others have previously reported that S1P-induced pulmonary endothelial barrier enhancement is associated with the formation of a prominent cortical actin ring (Garcia et al., 2001; Dudek et al., 2004; Shikata et al., 2003a,b; Singleton et al., 2005) coincident with FA remodeling. S1P induced the redistribution of FA proteins, paxillin and FAK, to the cell periphery, which was associated with paxillin tyrosine phosphorylation and selective FAK Y⁵⁷⁶ phosphorylation within the FAK catalytic domain (Shikata et al., 2003a,b). Actin cytoskeletal rearrangement is closely associated with FA distribution (Sastry and Burridge, 2000) via paxillin, a multi-domain adapter protein containing binding sites for various signaling molecules and structural proteins (Birge et al., 1993; Salgia et al., 1995; Turner and Milel, 1994; Turner et al., 1990; Weng et al., 1993; Wood et al., 1994). Paxillin facilitates signal transduction by recruiting specific molecules to FA, whereas the phosphorylation status of paxillin is an important determinant of binding partner selection (Bellis et al., 1995; Brown et al., 1998; Schaller and Parsons, 1995; Turner, 1998; Schaller, 2001; Brown and Turner, 2004). Together, our observations suggest that FAK and paxillin are active participants in S1P-induced FA remodeling and endothelial barrier enhancement, facilitating cortical actin ring formation.

Activation of Rac, a member of the family of small GTPases, is an essential driver of S1P-induced cortical actin ring formation (Garcia et al., 2001; Shikata et al., 2003y,b; Vouret-Craviari et al., 2002; Singleton et al., 2005). Rac regulates actin cytoskeletal remodeling and FA dynamics via ADP-ribosylation factor GTPase activating proteins (ARF GAPs) (Turner et al., 2001), which interact with several signaling and cytoskeletal proteins including paxillin. Among ARF GAP proteins, G-protein-coupled receptor kinase interacting protein 1 (GIT1) and Paxillin Kinase Linker or GIT2 (PKL/GIT2) directly bind paxillin and participate in signaling events including FA assembly and disassembly (Turner et al., 2001; Mazaki et al., 2001; West et al., 2001; Zhao et al., 2000). We have previously reported that S1P induced the transient increase in paxillin–GIT1 association and redistribution of both GIT1 and GIT2 to the cell periphery, suggesting the role of GITs in Rac-mediated cortical actin ring formation and redistribution of FA proteins (Shikata et al., 2003a,b). Furthermore, pharmacological inhibition of Src with Src-specific inhibitor PP2 abolished S1P-induced translocation of FA proteins, cortical actin ring formation, and FAK [Y⁵⁷⁶] phosphorylation (Shikata et al., 2003a,b). Recent data also indicated that the depletion of S1P₁ receptor using specific siRNA reduced the barrier protective effect of activated protein C (APC), suggesting the important roles of S1P₁ receptor activation in endothelial barrier enhancement coincident with cortical actin ring formation (Finigan et al., 2005).

Taken together, these data suggest a potential involvement of the S1P₁-receptor–Rac–Src signaling pathway in the redistribution of FAK and paxillin to the cell periphery coincident with selective FAK Y⁵⁷⁶ phosphorylation. Whereas focal adhesion remodeling plays a critical role in the S1P-induced endothelial barrier enhancement, the underlying mechanism facilitating the well coordinated redistribution of focal adhesion proteins to the cell periphery in endothelial cell monolayers remains to be elucidated. In the present paper, we studied S1P-induced interaction between adherens junction (AJ) and FA molecules, linked by β -catenin, as a potential novel mechanistic pathway in EC barrier regulation. Our results

suggest that pre-existing AJ protein complexes at cell–cell contacts mediate FA redistribution to the cell periphery via FA–AJ complex formation, and β -catenin plays a critical role in this process.

Methods

Reagents and antibodies

Chemicals and reagents, including synthetic S1P were obtained from Sigma Chemical (St. Louis, MO), unless noted otherwise. Fetal bovine serum (FBS) was obtained from CanSera International (Ont. Canada). Cell culture medium (EBM-2) and growth supplements were obtained from Clonetics (Walkersville, MD). Alexa Fluor 488 anti-mouse IgG antibody, Alexa Fluor 488 anti-rabbit IgG antibody and Texas red-phalloidin were purchased from Molecular Probes (Eugene, OR). Mouse monoclonal anti-FAK antibody, anti- β -catenin antibody and siIMPOR-TERTM siRNA transfection reagent were obtained from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal anti-paxillin antibody was obtained from BD Biosciences-Pharmingen (San Diego, CA). Rabbit polyclonal anti- α -catenin and mouse monoclonal anti-VE-cadherin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-linked anti-mouse and rabbit IgG antibodies and HRP Western blot detection kit were obtained from Amersham Biosciences (Piscataway, NJ). Protein G-Sepharose 4B conjugate was purchased from Zymed (San Francisco, CA).

Human pulmonary artery endothelial cell culture

Human pulmonary artery endothelial cells (HPAECs) were obtained from Lonza and cultured in EBM-2 complete medium containing 10% FBS. Endothelial cell cultures were maintained at 37 °C in a humidified atmosphere and grown to contact-inhibited monolayers with typical cobblestone morphology. Cells from each primary flask (passage 4 to 8) were detached with 0.05% trypsin, resuspended in fresh culture medium, and passaged into 60 mm² dishes for coimmunoprecipitation studies, or 12-well plates (with gelatinized coverslips) for immunofluorescent analysis.

Immunofluorescence microscopy

HPAECs were grown to 40% and 95% confluence for the observation of solitary cells and endothelial cell monolayer, respectively, on gelatinized coverslips. Cells were then rendered quiescent in EBM-2 containing 1% FBS for 20 h, incubated with 0.5 μ M S1P (diluted with medium from 1 mM S1P methanol stock solution) or vehicle control (same concentration of methanol), fixed in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, washed three times with PBS, permeabilized with 0.25% Triton X-100 in 0.1% Tween-20 containing Tris-buffered saline (TBS-T) for 15 min, and blocked with 2% BSA in TBS-T for 30 min. Incubations with primary antibodies of interest were performed in blocking solution (2% BSA in TBS-T) for 1 h at room temperature. After three washes with TBS-T, cells were incubated with appropriate secondary antibodies conjugated to Alexa 488 in blocking solution for 1 h at room temperature. Actin filaments were visualized by staining cells with Texas red-conjugated phalloidin for 1 h at room temperature. After three washes with PBS, the coverslips were mounted using Pristine Mount (Pharma, Tokyo). Analysis of immunofluorescent staining was performed using an Olympus IX71 microscope with $\times 40$ objective lens (Tokyo, Japan).

RNA interference experiments

For β -catenin depletion, siGENOME SMARTpoolTM CTNNB1 (Dharmacon, Chicago, IL), the mixture of β -catenin-specific siRNAs, was used. Non-specific control siRNA pool was

also obtained from Dharmacon. Non-specific control siRNA pool is the mixture of four RNA duplexes [5'-AUGAACGUGAAUUGCUCUAA (sense), 5'-UUGAG-CAAUUCACG-UUCAU (antisense), 5'-UAAGGCUAUGAAGAGAUAC (sense), 5'-GUAUCUC-UUCAUAGCCUUA (antisense), 5'-AUGUAUUG-GCCUGUAUUAG (sense), 5'-CUAAUACAGGCCAAUACA (antisense), 5'-UAGCGACUAAACACAUCUAA (sense), 5'-UUGAUGUGUUUAGUCG-CUA (antisense), respectively] with UU overhangs and a 5'-phosphate on the antisense strand. Transfection of siRNA was performed fundamentally according to the manufacturer's protocol. Briefly, HPAECs less than 40% confluent on gelatinized 6 well plates were incubated with 10% FCS-supplemented media containing siIMPORTER ($\times 200$), siIMPORTER ($\times 200$)/Control siRNA (200 nM) mixture and siIMPORTER ($\times 200$)/CTNNB1 (200 nM) mixture, respectively, and vehicle control for 48 h. Cells were then rendered quiescent in EBM-2 containing 1% FBS for 20 h before Western blot analysis or S1P stimulation followed by co-immunoprecipitation analysis.

Western blot analysis

After brief washing with PBS, cells prepared on 6 well dishes as described in the section of RNA interference experiments were lysed with 300 μ L per well of cell lysis buffer containing 10 mM Tris (pH 7.4), 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 0.2 mM vanadate, 0.2 mM PMSF, and 0.5% phosphatase inhibitor cocktail. Total cell lysates were cleared by centrifugation and the protein concentration was measured using DC protein assay kit (Sigma). Lysates were then boiled with the same amount of 3 $\times$ SDS sample buffer for 5 min and the same amount of proteins (6.5 μ g) were subjected to 7.5% SDS-PAGE. The separated proteins were transferred to PVDF membranes by electrotransfer. The blots were subsequently blocked with 5% FBS in TBS-T at room temperature for 1 h, then incubated at 4 $^{\circ}$ C overnight with primary mouse monoclonal anti-FAK (1:250 dilution), paxillin (1:1000 dilution), β -catenin (1:250 dilution) and VE-cadherin (1:250 dilution) antibodies and rabbit polyclonal anti- α -catenin (1:250 dilution). After washing three times for 10 min with TBS-T, the membrane was incubated with 1:1000 dilution of HRP-linked anti-mouse or rabbit IgG secondary antibody at room temperature for 1 h. The blots were then visualized with the ECL Western blot detection system. The amount of detected proteins was analyzed using Image Quant software.

Coimmunoprecipitation analysis

HPAECs grown on 60 mm² dishes were rendered quiescent in EBM-2 containing 1% FBS for 20 h, then stimulated with 0.5 μ M S1P dissolved in the same medium for the indicated time periods. After brief washing with PBS, cells were lysed with 600 μ L of coprecipitation buffer containing 50 mM Tris (pH 7.8), 1% NP-40 and 20 mM EDTA, 0.2 mM vanadate, and 0.2 mM PMSF (41) for a dish. Cells prepared on 6 well dishes as described in the section of transfection of siRNA against β -catenin were lysed with 300 μ L per well of coprecipitation buffer. For immunoprecipitation, 70 μ L of cell lysates was diluted with 100 μ L of the same buffer, then incubated with 2.5 μ g of the appropriate antibody (anti-paxillin, anti- α -catenin, anti- β -catenin or anti-VE-cadherin antibody) at 4 $^{\circ}$ C for 1 h, followed by incubation with protein G-sepharose 4B for 1 h. Sepharose beads were collected by centrifugation, washed three times with the same buffer, resuspended in 20 μ L of 3 $\times$ SDS sample buffer, then boiled for 5 min. Precipitated proteins were then separated as described for Western blot analysis. Resulted membranes were blotted with appropriate antibodies of interest (anti-paxillin (1:1000 dilution), anti-FAK anti- α -catenin, anti- β -catenin or anti-VE-cadherin (1:250 dilution) antibody). To reprobe membranes with antibodies against a precipitated protein, membranes were incubated in reprobing buffer containing 62.5 mM Tris (pH 6.8), 2% deoxycholate, and 100 mM mercaptoethanol at 50 μ M for 30 min. After being washed four times with TBS-T for 10 min, membranes were incubated with an

appropriate antibody (1:1000 dilution) at room temperature for 1 h, then visualized again. The amount of proteins was analyzed using Image Quant software.

Measurement of transendothelial electrical resistance

HPAECs were transfected by β -catenin siRNA (CTNNB1) or control siRNA. Measurements of transendothelial electrical resistance (TER) across confluent HPAEC monolayers treated with S1P were performed using an electrical cell-substrate impedance sensing system (ECIS) (Applied Biophysics, Troy, NY) as previously described in detail (Garcia et al., 2001; Birukova et al., 2007). The efficacy of β -catenin silencing and reductions in protein expression was assessed in the same group of cells by Western blot analysis.

Statistical analysis

Results are expressed as means $\pm$ SE of three to five independent experiments. Stimulated samples were compared with controls by unpaired Student's *t* test. For multiple group comparisons, one-way ANOVA (ANOVA) followed by the post hoc Fisher's test, was used. $P < 0.05$ was considered statistically significant.

Results

Differential redistribution of focal adhesion and adherens junction proteins induced by S1P in single cells and EC monolayers

S1P-induced actin reorganization and the redistribution of FAK and paxillin in ECs were monitored by immunofluorescent microscopy. In solitary quiescent EC, both FAK (Fig. 1A, panels a–c) and paxillin (Fig. 1B, panels a–c) were stained diffusely in the cytoplasm as well as at sites of stress fiber attachment to the randomly arranged FA. Incubation of solitary ECs with S1P (0.5 μ M, 30 min) did not cause peripheral redistribution of FAK (Fig. 1A, panels d–f) and paxillin (Fig. 1B, panels d–f) and cortical actin enhancement, which in contrast was observed in EC monolayers (Fig. 1A, panels g, h and Fig. 1B, panels g, h). Next, we analyzed distribution of AJ proteins and demonstrated that AJ proteins (VE-cadherin, α -catenin and β -catenin) stained diffusely and homogeneously in the cytoplasm of unstimulated solitary cells, and S1P challenge did not affect the distribution of these proteins (Figs. 1C, D and E). In contrast, S1P stimulation (0.5 μ M S1P, 30 min) caused recruitment of VE-cadherin, α -catenin and β -catenin to the cell periphery with enhancement of cell–cell contacts area (Figs. 1C, D and E).

S1P-induced alterations in VE-cadherin association with FAK and paxillin

We next explored whether S1P influences FAK and paxillin association with VE-cadherin. Quiescent HPAECs were stimulated with 0.5 μ M S1P for indicated time periods (0, 10 or 30 min), lysed and used in coimmunoprecipitation assays as described in Methods.

Interaction of VE-cadherin with FA proteins was analyzed by immunoblotting with antibodies to FAK and paxillin (Fig. 2). S1P challenge increased VE-cadherin association with FAK and paxillin within 10 min, which persisted at least 30 min.

S1P-induced alterations in VE-cadherin association with α -catenin and β -catenin

The effect of S1P on α -catenin and β -catenin association with VE-cadherin was also investigated. ECs were stimulated with S1P as described above followed by immunoprecipitation with VE-cadherin antibody. As shown in Fig. 3, S1P stimulation increased VE-cadherin association with both α -catenin and β -catenin within 10 min in EC monolayers and persisted at least 30 min. These results show an interaction of AJ protein VE-cadherin with α -catenin, β -catenin, FAK and paxillin. Importantly, S1P challenge

induced sustained increase in association of VE-cadherin with both AJ proteins and FA proteins (Figs. 2 and 3).

Differential effects of S1P on FAK association with α -catenin and β -catenin

To investigate interplay between α -catenin, β -catenin and FAK, and examine its possible involvement in S1P-mediated cell–cell contact remodeling, HPAECs were stimulated with 0.5 μ M S1P for indicated time periods (0, 10 or 30 min). Then, α -catenin and β -catenin were immunoprecipitated, and probed with antibody against FAK. Fig. 4 demonstrates that the amount of β -catenin coprecipitated with FAK significantly increased after S1P challenge, while the amount of α -catenin coprecipitated with FAK was not influenced.

Differential effects of S1P challenge on paxillin association with α -catenin and β -catenin

Next, using a similar experimental setup we studied interaction between paxillin and α - and β -catenin. Quiescent lung ECs were challenged with 0.5 μ M S1P for 0, 10 or 30 min, and paxillin immunoprecipitation performed. In contrast to the results of FAK analysis (Fig. 4), the amount of α -catenin coprecipitated with paxillin significantly increased after S1P challenge, while the amount of β -catenin coprecipitated with paxillin was unchanged (Fig. 5). These data demonstrate S1P-mediated increase in FAK– β -catenin and paxillin– α -catenin protein interactions and thus suggest specific interaction between FA and AJ proteins.

Effects of siRNA-based β -catenin protein depletion on S1P-induced VE-cadherin–FAK and VE-cadherin–paxillin complexes

In the next series of experiments, we downregulated β -catenin protein expression (siRNA) followed by Western blot analysis as described in Methods. Transfection with β -catenin-specific siRNA mixture (CTNNB1) resulted in substantial β -catenin depletion without changes in the protein expression of FAK, paxillin, α -catenin and VE-cadherin (Fig. 6). Cell treatment with siIMPORTER or transfection with control siRNA did not alter the protein expression of β -catenin and other related proteins (FAK, paxillin, α -catenin and VE-cadherin) (Fig. 6), indicating the direct effect of β -catenin-specific siRNAs on β -catenin depletion. ECs grown to 95% confluence were next transfected with β -catenin-specific siRNA, rendered quiescent for 20 h and then stimulated with 0.5 μ M S1P for 30 min. Immunoprecipitation of VE-cadherin was performed as described above followed by probing with FAK and paxillin antibodies. Coimmunoprecipitation study revealed that depletion of β -catenin resulted in the complete loss of VE-cadherin association with FAK and paxillin induced by 0.5 μ M S1P within 30 min (Figs. 7A, B).

To further examine S1P-induced barrier-protective effects through AJ/FA proteins in pulmonary EC, we downregulated endogenous β -catenin protein expression by transfecting β -catenin siRNA or control siRNA into HPAEC, and detected TER values in transfected HPAEC. Compared to cells transfected by control siRNA, depletion of β -catenin significantly attenuated S1P-mediated barrier protection (Fig. 8). Downregulation of β -catenin expression was confirmed by Western blot analysis in the same group of cells (Fig. 8, inset). These results indicate an essential role for AJ/FA proteins in the barrier-protective response of pulmonary EC to S1P.

Discussion

Despite recognition of the importance of FA remodeling in endothelial barrier regulation, the mechanisms facilitating S1P-induced distinctive redistribution of FAK and paxillin to the cell periphery remain to be elucidated. We explored the relationship between FA and AJ proteins as a potential mechanism of FA remodeling in response to S1P. We first analyzed FA and AJ redistribution in single cells as well as in human lung EC monolayers under

quiescent conditions and after S1P stimulation. Both FAK and paxillin were detected in FAs randomly distributed at the ends of actin-containing stress fibers in quiescent solitary cells (Figs. 1A and B). S1P challenge did not induce the formation of cortical actin ring coincident with dramatic redistribution of both FAK and paxillin to the cell periphery as was observed in HPAEC monolayers (Figs. 1A and B), whereas transient lamellipodia formation persisted. Furthermore, immunofluorescent staining of AJ proteins (α -catenin, β -catenin and VE-cadherin) revealed diffuse localization in the cytoplasm of solitary cells without marked alteration induced by S1P, whereas staining of a quiescent HPAEC monolayer with anti-VE-cadherin, α -catenin and β -catenin antibodies revealed strong distribution of these proteins within a cell–cell contact area, indicating intact and mature AJ formation (Figs. 1C–E). The findings shown in Fig. 1 strongly suggest that pre-existing AJ complexes at cell–cell contacts are critical and essential for the well-organized FAK and paxillin redistribution to a cell periphery.

Coimmunoprecipitation analysis showed S1P-induced association of VE-cadherin with FAK and paxillin (Fig. 2), which was consistent with immunofluorescence microscopy results (Fig. 1). These data strongly suggest the physical interaction of FA proteins with the core cadherin–catenin complex (Kobielak and Fuchs, 2004; Birukova et al., 2007). As shown in Fig. 3, S1P also induced the VE-cadherin association with α -catenin and β -catenin in HPAEC monolayers. These data are consistent with the previous observations showing S1P-induced β -catenin and VE-cadherin redistribution to cell–cell contact regions (Lee et al., 1999; Mehta et al., 2005; Xu et al., 2007). Endothelial cells adhere with each other via homophilic interaction of cadherin to maintain junction integrity (Bershadsky, 2004). β -Catenin binds directly to the cadherin cytoplasmic tail and recruits α -catenin into the protein complex, whereas p120-catenin binds to the membrane-proximal region of cadherin cytoplasmic tail independently of other catenins (Bershadsky, 2004; Kobielak and Fuchs, 2004). This complex of four proteins is commonly regarded as the core cadherin–catenin complex (Kobielak and Fuchs, 2004; Mehta et al., 2005). Our present data provide strong evidence for the S1P-induced enhancement of the core cadherin–catenin complex formation, possibly via providing additional docking sites for several signaling molecules and cytoskeletal proteins in the cell periphery by binding with catenins. Furthermore, S1P induced the association of FAK with β -catenin, but not with α -catenin (Fig. 4), while paxillin association with α -catenin but not with β -catenin was enhanced (Fig. 5). These coimmunoprecipitation results are also consistent with our previous findings that S1P-induced dissociation of FAK from paxillin correlated with the redistribution of both proteins to the cell periphery (Shikata et al., 2003a,b). Taken together, our present data strongly suggest the close association of FAK and paxillin with β -catenin and α -catenin, respectively, recruiting FAK and paxillin to both pre-existing and newly formed core cadherin–catenin complexes, which then facilitate the S1P-induced formation of FA–AJ complex in the cell cortical area. Depletion of β -catenin without significant influence on the expression of other proteins (FAK, paxillin, VE-cadherin and α -catenin) (Fig. 6) resulted in the complete loss of S1P-induced VE-cadherin association with FAK and paxillin (Fig. 7A). These findings again strongly suggest a role for FA–AJ interaction mediated by β -catenin in the endothelial barrier enhancement by S1P.

The mechanisms required to facilitate the enhancement of paxillin– α -catenin and FAK– β -catenin association remain to be elucidated. α -Catenin contains three vinculin homology (VH) domains in its structure, comprising a series of parallel four-helix bundles (Kobielak and Fuchs, 2004). VH1 domain is the docking site for β -catenin, while vinculin binds with VH3 domain of α -catenin (Kobielak and Fuchs, 2004) via four-helix bundle structure. Considering the association of paxillin-binding subdomain (PBS) of vinculin with leucine-rich domain (LD) motifs of paxillin (Brown and Turner, 2004), vinculin may mediate the indirect association between α -catenin and paxillin. Another possibility might exist for the

direct interaction between α -catenin and paxillin because the four-helix bundle structure in vinculin PBS is also shared by α -catenin (Brown and Turner, 2004). On the other hand, both the β -catenin-binding site of α -catenin and the FAK focal adhesion targeting (FAT) domain contain similar four-helix bundle structures, suggesting the possibility of direct interaction between FAK and β -catenin. Recent studies revealed that overexpression of a kinase-defective mutant FAK blocked hyperosmolar challenge-induced accumulation of both E-cadherin and F-actin in the cell periphery (Mitra et al., 2005; Quadri et al., 2003). These findings imply an important role of FAK in AJ kinetics, in which the direct FAK interaction with a member of core cadherin–catenin complex might be involved.

In summary, we have identified the augmented FA–AJ interaction in the barrier enhancement by S1P signaling. This demonstrates S1P-induced formation of a FA–AJ complex with a critical role of β -catenin in leading to the cortical actin ring arrangement. This study provides a mechanistic pathway underlying endothelial barrier enhancement and will drive future efforts to elucidate precise mechanisms of protein–protein interactions in FA–AJ complex formation.

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Fig. 1.

Differential distribution of focal adhesion and adherens junction proteins between solitary and monolayer-forming HPAECs. HPAECs grown on gelatinized coverslips were stained with anti-FAK (A), anti-paxillin (B), anti-VE-Cadherin (C), anti- α -catenin (D) and anti- β -catenin antibodies (E) as described in Methods. F-actin was also visualized with Texas red-phalloidin. Scale bars indicate 20 μ m.

Fig. 2.

S1P promotes the association of FAK and paxillin with VE-cadherin. HPAEC culture was stimulated with 0.5 μ M S1P for the indicated time periods and cell lysates were subjected to coprecipitation with anti-VE-cadherin antibody. (A) FAK was detected in immunoprecipitates as described in Methods. Reprobing analysis of membranes with anti-VE-cadherin antibody show equal amounts of VE-cadherin precipitated and loaded. Representative blots of 6 independent experiments are shown. (B) Paxillin was detected in VE-cadherin immunoprecipitates. Equal amounts of VE-cadherin were precipitated and loaded. Representative blots of 6 independent experiments are shown. (C) Amounts of coprecipitated proteins (FAK and paxillin) were quantified and analyzed statistically. Values indicate the amount (%) of coimmunoprecipitated proteins at various time points assuming the 0 min value as 100% (mean $\pm$ SE, * p <0.0001, ** p <0.01).

Fig. 3.

α -catenin and β -catenin with VE-cadherin. HPAEC culture was stimulated with 0.5 μ M S1P for the indicated time periods and cell lysates were subjected to coprecipitation with anti-VE-cadherin antibody. (A) α -Catenin was detected in immunoprecipitates as described in Methods. Reprobing analyses of membranes with anti-VE-cadherin antibody show equal amounts of VE-cadherin precipitated and loaded. Representative blots of 6 independent experiments are shown. (B) β -Catenin was detected in immunoprecipitates as described in Methods. Equal amounts of VE-cadherin were precipitated and loaded. Representative blots of 6 independent experiments are shown. (C) Amounts of coprecipitated proteins (α -catenin and β -catenin) over time were quantified and analyzed statistically. Values indicate the amount (%) of coimmunoprecipitated proteins at various points assuming the 0 min value as 100% (mean $\pm$ SE, * $p < 0.05$, ** $p < 0.01$).

Fig. 4.

S1P promotes the association of FAK with β -catenin, but not with α -catenin. (A) HPAEC culture was stimulated with 0.5 μ M S1P for the indicated time periods and cell lysates were subjected to coprecipitation with anti- α -catenin antibody. Resulting membranes were probed with anti-FAK antibody as described in Methods. Reprobing analyses of membranes with anti- α -catenin antibody revealed equal amounts of proteins precipitated and loaded by lanes. Representative blots of 6 independent experiments are shown. (B) Cell lysates from HPAECs stimulated with 0.5 μ M S1P for the indicated periods of time were subjected to coprecipitation with anti- β -catenin antibody. Resulting membranes were probed with anti-FAK antibody as described in Methods. Reprobing analyses of membranes with anti- β -catenin antibody indicated equal amount of proteins precipitated and loaded by lanes. Representative blots of 6 independent experiments are shown. (C) Amounts of coprecipitated FAK with α -catenin and β -catenin were quantified and analyzed statistically. Values indicate the amount (%) of coimmunoprecipitated FAK at various points assuming the 0 min value as 100% (mean $\pm$ SE, * $p < 0.01$, ** $p < 0.0001$).

Fig. 5.

S1P promotes the association of paxillin with α -catenin, but not with β -catenin. HPAEC culture was stimulated with 0.5 μ M S1P for the indicated time periods and cell lysates were subjected to coprecipitation with anti-paxillin antibody. (A) α -Catenin was detected in immunoprecipitates as described in Methods. Representative blots of 6 independent experiments including reprobing analyses of membranes with anti-paxillin antibody are shown. (B) β -Catenin was detected in immunoprecipitates, and the membranes were then reprobed with anti-paxillin antibody. Representative blots of 6 independent experiments are shown. (C) Normalized amounts of coprecipitated proteins (α -catenin and β -catenin) over time were quantified and analyzed statistically. Values indicate the amount (%) of coimmunoprecipitated proteins at various points assuming the 0 min value as 100% (mean $\pm$ SE, * p <0.01, ** p <0.001).

Fig. 6.

Transfection with β -catenin-specific siRNA does not influence the protein expression of FAK, paxillin VE-cadherin and α -catenin. (A) siRNA transfection against β -catenin into HPAECs and the following Western blot analysis with anti-FAK, paxillin VE-cadherin, α -catenin and β -catenin were performed as described in Methods. Representative blots of 3 independent experiments are shown. (B) Amounts of FAK, paxillin, VE-cadherin, α -catenin and β -catenin were quantified and analyzed statistically. The transfection of β -catenin specific siRNA mixture (CTNNB1) to HPAECs resulted in the remarkable β -catenin depletion ($33.4 \pm 5.0\%$ of control) without any significant change in the protein expression of FAK, paxillin, α -catenin and VE-cadherin. Treatment of cells with siIMPORTER or transfection of control siRNA did not alter the protein expression of β -catenin and other related proteins (FAK, paxillin, α -catenin and VE-cadherin). Values indicate the amount (%) of proteins assuming the control value as 100% (mean $\pm$ SE, $*p < 0.0001$).

Fig. 7.

Inhibitory effects of β -catenin depletion on VE-cadherin-FAK and -paxillin complex induced by S1P challenge. EC were transfected with β -catenin specific siRNA or control siRNA as described in Methods. Both transfected and non-transfected ECs on 6-well plates were rendered quiescent for 20 h and then stimulated with 0.5 μ M S1P for 30 min. Cell lysates were then subjected to coprecipitation with anti-VE-cadherin antibody. (A) FAK and paxillin were detected in immunoprecipitates as described in Methods. Representative blots of 3 independent experiments including reprobing analyses of membranes with anti-VE-cadherin antibody are shown. (B) Normalized amounts of coprecipitated proteins (FAK and paxillin) were quantified and analyzed statistically. Values indicate the amount (%) of coimmunoprecipitated proteins assuming the 0 min control value as 100% (mean $\pm$ SE, * p <0.001, ** p <0.0001, *** p <0.01).

Fig. 8.

Depletion of β -catenin attenuates S1P induced-barrier enhancement in human lung endothelium. Human lung endothelial cells (HPAECs) were transfected with 100 nM β -catenin specific siRNA or control siRNA. After 48 h, cells were trypsinized and seeded in polycarbonate wells containing evaporated gold microelectrodes. After 24 h, cells became confluent and TER measurements were then performed using an electrical cell-substrate impedance sensing system (ECIS) in siRNA transfected HPAEC with or without 1 μ M S1P stimulation. β -Catenin depletion induced by β -catenin specific siRNA was confirmed by Western blot (*inset*). Results are representative of 3 independent experiments.