Cyclic AMP Potentiates Vascular Endothelial Cadherin-Mediated Cell-Cell Contact To Enhance Endothelial Barrier Function through an Epac-Rap1 Signaling Pathway

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Cyclic AMP (cAMP) is a well-known intracellular signaling molecule improving barrier function in vascular endothelial cells. Here, we delineate a novel cAMP-triggered signal that regulates the barrier function. We found that cAMP-elevating reagents, prostacyclin and forskolin, decreased cell permeability and enhanced vascular endothelial (VE) cadherin-dependent cell adhesion. Although the decreased permeability and the increased VE-cadherin-mediated adhesion by prostacyclin and forskolin were insensitive to a specific inhibitor for cAMP-dependent protein kinase, these effects were mimicked by 8-(4-chlorophenylthio)-2′,2′,5′-trimethyladenosine-3′,5′-cyclic monophosphate, a specific activator for Epac, which is a novel cAMP-dependent guanine nucleotide exchange factor for Rap1. Thus, we investigated the effect of Rap1 on permeability and the VE-cadherin-mediated cell adhesion by expressing either constitutive active Rap1 or Rap1GAPII. Activation of Rap1 resulted in a decrease in permeability and enhancement of VE-cadherin-dependent cell adhesion, whereas inactivation of Rap1 had the counter effect. Furthermore, prostacyclin and forskolin induced cortical actin rearrangement in a Rap1-dependent manner. In conclusion, cAMP-Epac-Rap1 signaling promotes decreased cell permeability by enhancing VE-cadherin-mediated adhesion lined by the rearranged cortical actin.

Endothelial cells lining blood vessels regulate endothelial barrier function, which restricts the passage of plasma proteins and circulating cells across the endothelial cells. Endothelial barrier dysfunction results in an increase in vascular permeability, thereby causing edema or inflammatory or metastatic cell infiltration. Inflammatory mediators such as thrombin and histamine induce intercellular gap formation, leading to an increase in endothelial permeability (1, 4). In contrast, angiopoietin 1 and sphingosine-1-phosphate (SIP) stabilize endothelial barrier integrity (17, 18). In addition, cyclic AMP (cAMP), a second messenger downstream of Gs-coupled receptor, improves endothelial cell barrier function (32, 39, 43). Consistently, cAMP-elevating G protein-coupled receptor (GPCR) agonists, adrenomedullin (AM), prostacyclin (PGI2), prostaglandin E2 (PGE2), and β-adrenergic agonists reduce endothelial hyperpermeability induced by inflammatory stimuli (15, 19, 25).

The endothelial cell barrier is structurally organized by adherens junctions (AJ) and tight junctions. Vascular endothelial (VE) cells express both VE-cadherin (also known as cadherin-5 and CD144) and neural (N)-cadherin (9, 33). VE-cadherin constitutes AJ, whereas N-cadherin formed the cell-cell contacts between endothelial cells and endothelial cell-supporting pericytes. VE-cadherin mediates calcium-dependent, homophilic intercellular adhesion. Its short cytoplasmic tail binds to three armadillo family proteins, β-, γ- and p120-catenins. β- and γ-catenins associated with α-catenin link the VE-cadherin complex to the actin cytoskeleton and, therefore, strengthen the AJ adhesiveness (9).

Endothelial AJ are dynamic structures, and their adhesive property is finely regulated by several different mechanisms. Tyrosine phosphorylation of VE-cadherin, β-catenin, and p120-catenin correlates with weakened endothelial cell-cell adhesion. VE growth factors and inflammatory mediators such as histamine and thrombin induce tyrosine phosphorylation of AJ components, resulting in the weakened cell-cell contacts and increased endothelial cell permeability (1, 14, 40). In clear contrast, angiopoietin 1, which stabilizes cell-cell contacts, induces dephosphorylation of endothelial cell adhesion molecules, VE-cadherin, and platelet endothelial cell adhesion molecule 1 (17). It has been also reported that SIP induces AJ formation and enhances barrier function through a Rac-dependent cortical actin rearrangement (18). cAMP-dependent protein kinase A (PKA) is suggested to be crucial for cAMP-triggered stabilization of cell-cell contacts and for barrier integrity of endothelial cells (43). However, it has not been clear whether PKA-independent signaling is involved in the regulation of endothelial barrier function.

Rap1, belonging to Ras family GTPase, is involved in the formation and stabilization of AJ in Drosophila melanogaster (23). Rap1 becomes the GTP-bound active form by guanine...
nucleotide exchange factor (GEF) and the GDP-bound inactive form by GTPase-activating proteins (GAP), respectively. GEFs for Rap1 include C3G, CalDAG-GEFs, Epacs, and DOCK4 (reviewed in reference 6). DOCK4, which is disrupted in various types of human cancers, regulates the formation of AJ (41). Very recent reports also revealed that Rap1 activity is required for the formation of E-cadherin-based cell-cell contacts (20, 36). These findings prompted us to investigate how Rap1 is activated to stabilize cell-cell contacts and to examine the physiological consequence of stabilized cell-cell contacts by Rap1.

In the present study, we investigated the mechanism by which cAMP-elevating GPCR agonists potentiate endothelial barrier function and restrict cell permeability. We found that increased CAMP triggers Epac-Rap1 signaling to reduce permeability independently of PKA by augmentation of VE-cadherin-mediated cell-cell adhesion.

MATERIALS AND METHODS

Reagents and antibodies. Human recombinant AM was kindly provided by Shionogi & Co. Ltd. (31). Materials were purchased as follows: isoprotenerol (Iso), PGE2, PGZ2, thrombin, forskolin (FSK), and 3-isotubyl-1-methylxanthine (IBMX) from Wako Pure Chemical Industries; dibutyryl-cAMP (dbcAMP) from Sigma-Aldrich; H89 from Sekigakus Corporation; 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cylic monophosphate (8-CPT-2′-O-Me-cAMP) from Tocris; fluorescein isothiocyanate (FITC)-labeled dextran (molecular weight, 42,000) and purified human immunoglobulin G (IgG) Fc protein from ICN Biocentials; vascular endothelial growth factor (VEGF) from R & D Systems. Anti-Rap1GAP11 antibody was developed by immunization of glutathione S-transferase (GST)-tagged Rap1GAP11 (amino acids 411 to 694 of Rap1GAP11). Other antibodies used here were purchased as follows: anti-VE-cadherin from Chemicon International and Transduction Laboratories; anti-p-catenin from Transduction Laboratories; anti-CREB and anti-phospho-CREB (Ser133) from Cell Signaling Technology; anti-Rap1 from Santa Cruz Biotechnology; anti-cortactin from Upstate Biotechnology, Inc.; rhodamine-phalloidin and Alexa 488-labeled goat anti-mouse IgG from Molecular Probes; horseradish peroxidase-coupled goat anti-mouse and goat anti-rabbit IgG from Amersham Biosciences.

Cell culture and transfection. Human umbilical vein endothelial cells (HUVECs) and human arterial endothelial cells (HAECs) were purchased from Kurabo (Kurashiki, Japan). The arterial cells were maintained in HuMedia-E22 (Kuraray, Osaka, Japan), and the umbilical cells in HuMedia-E22 which is a modified E-22 medium (HuMedia-E22 was modified by the addition of 0.1% bovine serum albumin and 2% heat-inactivated fetal calf serum). HUVECs and HAECs were grown to confluence in a six-well plate and serum starved in medium 199 containing 1% BSA overnight. The cells were stimulated with PGI2 and FSK for 30 min and incubated with cAMP in the absence of at least 2 min. The cultures were fixed with 4% formaldehyde in PBS containing 0.5% Triton X-100 for 15 min at 4°C and permeabilized with 0.2% Triton X-100 prior to staining. The cells were incubated with blocking buffer (PBS containing 1% BSA) for 30 min, and then the primary antibodies were added to the cultures for 1 h at 37°C. The cells were washed with PBS three times and then incubated with species-matched peroxidase-conjugated secondary antibodies. Immunocomplexes were visualized by enhanced chemiluminescence detection (Amersham Biosciences) with horseradish peroxidase-conjugated secondary antibodies.

Permeability assay. Permeability across the endothelial cell monolayer was measured by using type I collagen-coated transwell units (6.5-mm diameter, 3.0-μm-pore-size polycarbonate filter; Corning Costar Corporation). HUVECs plated at 105 cells in each well were cultured for 3 to 4 days before experiments. After serum starvation in medium 199 containing 1% bovine serum albumin (BSA) for 1 h, the cells were treated with the agonists or drugs, as indicated in the figure legends, for 30 min. Permeability was measured by adding 1 μg of FITC-labeled dextran (molecular weight, 42,000)/ml together with or without 2 U of thrombin/ml to the upper chamber. After incubation for 30 min, 50 μl of sample from the lower compartment was diluted with 300 μl of phosphate-buffered saline (PBS) and measured for fluorescence at 520 nm when excited at 492 nm with a spectrophotometer F-4500 (Hitachi). HUVECs infected with adenovirus for 24 h after becoming confluent and kept for another 24 h in replaced medium were subjected to a cell permeability assay.
agonists and drugs and lysed at 4°C in a pull-down lysis buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM MgCl2, 1% Triton X-100, 1 mM EGTA, 1 mM Na3VO4, 1 mM dithiothreitol, 1 mM)

HUVECs grown on transwell filters were incubated with control layer-cultured HUVECs as described in Materials and Methods. Measuring the fluorescence of FITC-labeled dextran across the monolayer-cultured HUVECs was analyzed by HUVECs. Expectedly, AM, Iso, PGE2, and PGI2 reduced thrombin-induced permeability (data not shown). The bio-ligands for cAMP-elevating GPCR that we used in this study indeed increased cAMP in HUVECs (data not shown). Furthermore, IBMX (an inhibitor for phosphodiesterase), dbcAMP (a membrane-permeable cAMP analogue), and FSK (an adenylyl cyclase activator) resulted in a reduction of both basal and thrombin-induced endothelial permeability (Fig. 1; data not shown).

cAMP potentiates formation of AJ. Endothelial barrier function is largely dependent upon endothelial cell junctions. To investigate how cAMP affects AJ formation, we examined AJ organization by immunostaining with anti-VE-cadherin before and after stimulation. When subconfluent HUVECs with intercellular gaps were stimulated with PGI2 or FSK, the cells extended the plasma membrane and established cell-cell contacts with neighboring cells (Fig. 2A). Similar results were

RESULTS

cAMP enhances the barrier property of monolayer-cultured endothelial cell. To evaluate the barrier function, we examined the permeability of FITC-labeled dextran across monolayer-cultured HUVECs. Expectedly, AM, Iso, PGE2, and PGI2 reduced basal endothelial permeability in HUVECs (Fig. 1A). PGI2 also reduced thrombin-induced vascular permeability (Fig. 1B). Other cAMP-elevating bio-ligands similarly reduced thrombin-induced permeability (data not shown). The bio-ligands for cAMP-elevating GPCR that we used in this study indeed increased cAMP in HUVECs (data not shown). Furthermore, IBMX (an inhibitor for phosphodiesterase), dbcAMP (a membrane-permeable cAMP analogue), and FSK (an adenylyl cyclase activator) resulted in a reduction of both basal and thrombin-induced endothelial permeability (Fig. 1; data not shown).

In vivo permeability assay. In vivo permeability was quantified by a modified Miles assay as described previously (29). In brief, ICR mice (Japan SLC, Inc.) shaved 3 days before experiments were lightly anesthetized and intravenously injected with 150 l of 1% Evans blue dye solution (in saline) passed through a 0.22-μm-pore-size filter. Fifteen minutes later, 20 l of PBS, VEGF (50 ng/ml), and/or 8-CPT-2′-O-Me-cAMP (1 μM) were applied by intradermal injections with vehicle (top), 10 μM FSK for 45 min were fixed, stained with anti-VE-cadherin antibody, and visualized with Alexa 488-conjugated secondary antibody through a confocal microscope (BX51WI, Olympus). Note that VE-cadherin (green) was accumulated at the cell-cell contact upon PGI2 and FSK stimulation. Bars, 50 μm. (C) Translocation of VECadherin was assessed by Triton X-100 solubility. HUVECs were stimulated with vehicle (top), 10-μg/ml PGI2 (middle), and 10 μM FSK (bottom) for the time indicated at the top and fractionated with cytoskeleton-stabilizing buffer as described in Materials and Methods. The Triton X-100-insoluble fraction was subjected to SDS-PAGE followed by Western blot analysis (WB) with anti-VE-cadherin.

FIG. 1. cAMP enhances barrier function of monolayer VE cells. (A) Vascular permeability, reflecting barrier function, was analyzed by measuring the fluorescence of FITC-labeled dextran across the monolayer-cultured HUVECs as described in Materials and Methods. HUVECs grown on transwell filters were incubated with control (Cont), 0.1 μM AM, 200 μM ISO, 200-ng/ml PGE2, 10-μg/ml PGI2, 1 mM IBMX, 1 mM dbcAMP, and 10 μM FSK for 30 min. Average permeability ± standard deviation is expressed as a percentage compared to the control. (B) The effects of PGI2 and FSK on vascular permeability were quantified in the presence (+) or absence (−) of vehicle (2 U of thrombin (Thr)/ml). Average permeability ± standard deviation is expressed as the increase relative to that observed in unstimulated HUVECs in the vehicle. Data shown are the results from at least three independent experiments. Significant differences from the control (A) or between two groups (B) determined by Student’s t test are indicated by a single asterisk (P < 0.05) or double asterisks (P < 0.01).
obtained with AM and PGE2 (data not shown). Stimulation of HUVECs with PGI2 and FSK dramatically enhanced accumulation of VE-cadherin at cell-cell contacts (Fig. 2B).

The maturation of AJ requires homophilic binding of intercellular VE-cadherins and tight anchoring to the actin cytoskeleton via the cytoplasmic region through catenins. VE-cadherin anchored to the actin cytoskeleton is detected in detergent-insoluble fractions of cell lysates (26). We found an increase in VE-cadherin in the Triton X-100-insoluble fraction after stimulation with PGI2 or FSK (Fig. 2C). These results suggest that cAMP-elevating GPCR agonists potentiate AJ formation, which results in a cAMP-induced decrease in permeability.

cAMP promotes VE-cadherin-dependent endothelial cell adhesion. VE-cadherin is required for AJ formation (9). To test the involvement of a homophilic interaction of VE-cadherin in cAMP-enhanced AJ formation, we directly examined VE-cadherin-mediated cell adhesion. To mimic the VE-cadherin-dependent cell adhesion, we used VEC-Fc chimeric protein, which consisted of the extracellular domain of VE-cadherin fused to the Fc portion of immunoglobulin. HUVECs were plated onto VEC-Fc-coated dishes and time-lapse imaged. Cells attached within 5 min to the VEC-Fc-coated dish, subsequently spread, and exhibited a typical fried-egg morphology characterized by a large circular lamellipodium (Fig. 3A). No cells attached to the Fc-coated dish (Fig. 3B and C). Since cadherin-dependent cell adhesion requires Ca\(^{2+}\), we examined the effect of Ca\(^{2+}\) chelation on cell adhesion to VEC-Fc-coated dishes. Cell adhesion to VEC-Fc-coated dishes was completely abolished by chelating extracellular Ca\(^{2+}\), although cell attachment to the collagen-coated dish was unaffected (Fig. 3C and D). Basal and FSK-augmented cell adhesion to VEC-Fc-coated dishes was inhibited by EGTA (Fig. 3C). Both HUVECs and HAECs expressing VE-cadherin adhered to the VEC-Fc-coated dish (Fig. 3E). In clear contrast, HeLa and HEK293 cells, which express N-cadherin, but not VE-cadherin (20, 42), did not adhere to the VEC-Fc-coated dish, although these cells could attach to the collagen-coated dish (Fig. 3E; data not shown). Collectively, these results indicate that endothelial cell adhesion to the VEC-Fc-coated dish depends upon the homophilic ligation of VE-cadherin.

We proceeded to investigate the effect of cAMP-elevating GPCR agonists on VE-cadherin-mediated cell adhesion. The adhesion of HUVECs plated in the presence of PGI2 or FSK was evaluated by the alkaline phosphatase activity of remaining cells after washing. PGI2 enhanced adhesion of HUVECs to the VEC-Fc-coated dish in a concentration-dependent manner (Fig. 4A) and in a time-dependent manner (Fig. 4B). In time course analysis, we noticed that enhanced adhesion was observed 7 min after the plating (Fig. 4B). Other cAMP-elevating GPCR agonists, including AM, Iso, and PGE2, potentiated VE-cadherin-dependent cell adhesion (Fig. 4C). In addition, similarly enhanced cell adhesion to the VEC-Fc-coated dish was also observed in the cells treated with cAMP-elevating drugs such as IBMX, dbcAMP, and FSK (Fig. 4F). Like PGI2, the effect of FSK on cell adhesion to the VEC-Fc-coated dish was concentration dependent and time dependent (Fig. 4D and E). This cAMP-induced cell adhesion to the VEC-Fc-coated dish depends on the enhanced homophilic ligation of VE-cadherin because FSK did not augment endothelial adhe-
adhesion activity was quantified as described in Materials and Methods. Mean
PGI2 at the concentrations indicated at the bottom for 7 min. Cell
(A) HUVECs were plated onto a VEC-Fc-coated dish in the presence
ence (square) of 10–g/ml PGI2 for the time indicated at the bottom.
/H9262 plated onto the VEC-Fc-coated dish in the absence (circle) or pre-
compared with that observed in unstimulated cells. (B) HUVECs were
stimulated with the reagent indicated at the bottom for 7 min. Cell
were preincubated for 10 min before plating. (E) The effect of 10 μM
FSK on time-dependent adhesion was analyzed as described in the
legend to panel B, except that cells were preincubated for 10 min
before plating. (F) HUVECs stimulated with the reagent indicated at
the same concentration used as described in the legend to Fig. 1A were
analyzed for cell adhesion by a method similar to that described for
panel D. Data are expressed as means ± standard deviations of the
results from three independent experiments in panels A, C, D, and F.
 Representative results from three independent experiments were
shown in panels B and E. A significant difference from the control
determined by Student’s t test is indicated with a single asterisk (P <
0.05) or double asterisks (P < 0.01).

FIG. 4. cAMP potentiates VE-cadherin-dependent cell adhesion.
(A) HUVECs were plated onto a VEC-Fc-coated dish in the presence
of PGI2 at the concentrations indicated at the bottom for 7 min. Cell
adhesion was quantified as described in Materials and Methods. Mean
adhesion activity ± standard deviation is expressed as the increase
compared with that observed in unstimulated cells. (B) HUVECs were
plated onto the VEC-Fc-coated dish in the absence (circle) or pres-
stimulated with cAMP-elevating ligands similar to that described in the
legend to Fig. 1A. (D) The effect of FSK on cell adhesion was analyzed
by a method similar to that described for panel A, except that cells
were preincubated for 10 min before plating. (E) The effect of 10 μM
FSK on time-dependent adhesion was analyzed as described in the
legend to panel B, except that cells were preincubated for 10 min
before plating. (F) HUVECs stimulated with the reagent indicated at
the same concentration used as described in the legend to Fig. 1A were
analyzed for cell adhesion by a method similar to that described for
panel D. Data are expressed as means ± standard deviations of the
results from three independent experiments in panels A, C, D, and F.
Representative results from three independent experiments were
shown in panels B and E. A significant difference from the control
determined by Student’s t test is indicated with a single asterisk (P <
0.05) or double asterisks (P < 0.01).
8-CPT-2’-O-Me-cAMP dramatically reduced basal endothelial permeability, as did FSK and dbcAMP (Fig. 7B). Thrombin-induced permeability was also inhibited by 8-CPT-2’-O-Me-cAMP (Fig. 7C). Furthermore, we examined the effect of 8-CPT-2’-Me-cAMP on in vivo vascular permeability. VEGF-induced vascular permeability was completely blocked by coinjection of 8-CPT-2’-O-Me-cAMP (Fig. 7D). In addition, adhesion

FIG. 5. cAMP-enhanced VE-cadherin-dependent cell adhesion and endothelial barrier function does not depend upon PKA. (A) Permeability across monolayer HUVECs grown on transwell filters were assessed by measuring FITC-labeled dextran as described in the legend to Fig. 1A. The effect of 10-µg/ml PGI2 on cell permeability without pretreatment (Vehicle) or with pretreatment with 5 µM H89, a specific PKA inhibitor, for 10 min is indicated as the percent permeability compared to that observed in untreated cells. +, present; −, absent. (B) The effect of 10 µM FSK on cell permeability without pretreatment (Vehicle) and with pretreatment with H89 was assessed similar to that described for panel A. (C) The effect of pretreatment of HUVECs with 5 µM H89 on FSK-induced reduction of 2-U/ml thrombin-induced permeability was analyzed. Permeability indicates the increase relative to that observed in untreated cells. (D) HUVECs untreated or pretreated with H89 for 10 min prior to stimulation with 10-µg/ml PGI2 were analyzed for cell adhesion as described in the legend to Fig. 4A. (E) The effect of pretreatment of H89 on cell adhesion was assessed by a method similar to that described for panel A. (F) The effect of 10 µM FSK on time-dependent Rap1 activity was examined as described for panel C. Representative results from at least three independent experiments are shown for all panels.

FIG. 6. cAMP induces Rap1 activation. (A) Serum-starved HUVECs kept in medium 199 containing 1% BSA overnight were stimulated with cAMP-elevating agonists for 2.5 min as indicated at the top and at the concentrations described in the legend to Fig. 1A. GTP-bound Rap1 was detected by pull-down assay as described in Materials and Methods. Activation indicates the ratio of the poststimulation GTP-Rap1 intensity of total Rap1 intensity to the prestimulation GTP-Rap1 intensity of total Rap1 intensity. (B) Rap1 activation was analyzed by detecting GTP-bound Rap1 with lysates from HUVECs stimulated with PGI2 for 2.5 min at the different concentrations indicated at the top. (C) Rap1 activation was analyzed by detecting GTP-bound Rap1 with lysates from cells stimulated with 10-µg/ml PGI2 for the time period indicated at the top. (D) Serum-starved HUVECs similar to those described in the legend to panel A were stimulated with the reagents indicated at the top for 10 min at the same concentrations described in the legend to Fig. 1A. Rap1 activation was assessed by a method similar to that described for panel A. (E) The effect of 10 µM FSK on time-dependent Rap1 activity was examined as described for panel C. Representative results from at least three independent experiments are shown for all panels.

by Student’s t test are indicated by a single asterisk (P < 0.05) or double asterisks (P < 0.01). (F) HUVECs serum starved in 1% BSA-containing medium 199 for 6 h, followed by pretreatment with (+) or without (−) 5 µM H89 for 10 min, were stimulated with vehicle and 10 µM FSK for 10 min. Phosphorylation of CREB was assessed by Western blot analysis with anti-CREB (CREB) and anti-phospho-CREB-specific (pCREB) antibodies.
of HUVECs to the VEC-Fc-coated dish was significantly enhanced by 8-CPT-2'-O-Me-cAMP (Fig. 7E). Hence, Epac activation is sufficient to enhance VE-cadherin-dependent cell adhesion and to augment endothelial barrier function in vitro and in vivo.

**Rap1 activation is essential for VE-cadherin-dependent cell adhesion and endothelial barrier function.** We next proceeded to investigate the role of Rap1 in VE-cadherin-dependent cell adhesion and endothelial barrier function. To examine the effect of Rap1 on cell permeability and VE-cadherin-mediated cell adhesion, we inactivated endogenous Rap1 by adenovirus-expressing Rap1GAPII (Ad-RapGAP), which specifically catalyzes the hydrolysis of GTP to GDP on Rap1 (30). As shown in Fig. 8A, endogenous Rap1 activity was almost completely suppressed by the expression of increasing amounts of Rap1GAPII in HUVECs. This Rap1 inactivation paralleled the increase in basal permeability (Fig. 8B) and the inhibition of cell adhesion to the VEC-Fc-coated dish (Fig. 8D). In contrast, a constitutively active Rap1, Rap1V12, reduced both basal and thrombin-increased cell permeability (Fig. 8C). VE-cadherin-mediated cell adhesion was also enhanced by Rap1V12 and EpacΔcAMP, a constitutively active mutant of Epac (Fig. 8D). Taken together, these results indicate that Rap1 activation is required for VE-cadherin-mediated cell adhesion and endothelial barrier function.

cAMP enhances VE-cadherin-dependent cell adhesion and endothelial barrier function by activating Rap1. To test the requirement for Rap1 in endothelial barrier enhancement by cAMP-elevating GPCR agonists, we infected HUVECs with Ad-RapGAP and examined the effect of inactivation of Rap1 on PGI2- and FSK-induced reduction of cell permeability. Although basal endothelial permeability was reduced by PGI2 and FSK (Fig. 9A and B), overexpression of Rap1GAPII increased not only basal but also PGI2- and FSK-reduced endothelial permeability, indicating the requirement of Rap1 activity for PGI2- and FSK-induced barrier enhancement. We also investigated the involvement of Rap1 in PGI2- and FSK-induced VE-cadherin-dependent cell adhesion. PGI2 and FSK augmented VE-cadherin-dependent cell adhesion of HUVECs infected with control adenovirus (Ad-LacZ); however, their effects were dramatically suppressed by overexpression of Rap1GAPII (Fig. 9C and D). These data demonstrate that cAMP enhances VE-cadherin-dependent cell adhesion and endothelial barrier functions by activating Rap1.

**cAMP induces endothelial cortical actin rearrangement in a Rap1-dependent manner.** Endothelial barrier function is largely dependent upon the actin cytoskeleton supporting junctional adhesion molecules (10). Thus, we examined the effect of cAMP on cortical actin polymerization and assembly of polymerized actin in a monolayer of endothelial cells. Cortactin, an actin-binding protein, is known to be implicated in cortical actin rearrangement (8) and suggested to regulate S1P-induced endothelial barrier enhancement (11). PGI2, 7 min was analyzed as described in the legend to Fig. 4F. In panels B, C, and E, data are expressed as means ± standard deviations of the results from triplicate samples. A significant difference from the control in panels B and E or between two groups in panels C and D was determined by Student’s t test and indicated by a single asterisk (*P < 0.05) or double asterisks (**P < 0.01).
FSK, and 8-CPT-2’-O-Me-cAMP dramatically induced accumulation of polymerized actin and cortactin at cell-cell contacts (Fig. 10A). To explore the involvement of Rap1 in cAMP-mediated cortical actin rearrangement, an expression vector encoding Rap1GAPII was introduced into endothelial cells. FSK enhanced actin polymerization at cell-cell contacts in cells transfected with control vector encoding EGFP, whereas it did not in cells expressing Rap1GAPII (Fig. 10B). Cytochalasin D, an actin-depolymerizing agent, attenuated FSK-induced barrier enhancement (Fig. 10C) and inhibited FSK-induced VE-cadherin-dependent cell adhesion (Fig. 10D). These results suggest that the cortical actin rearrangement promoted by...
cAMP-Epac-Rap1 signaling may contribute to the potentiation of endothelial barrier function and VE-cadherin-dependent cell adhesion.

**DISCUSSION**

cAMP is a well-known intracellular signaling molecule that is capable of restoring diminished endothelial barrier function. Previous reports suggested that cAMP-induced barrier enhancement occurs through PKA (27, 39). In this study, however, we demonstrated a novel PKA-independent signaling pathway, the cAMP-Epac-Rap1 signaling pathway, involved in cAMP-induced barrier function based on the following observations. PGI2- and FSK-reduced endothelial permeability was insensitive to H89. A specific activator for Epac, 8-CPT-2'-O-Me-cAMP, reduced both basal and thrombin-increased permeability. Plasma leakage in response to VEGF was also inhibited by 8-CPT-2'-O-Me-cAMP in vivo. We found that the activation of Rap1 leads to decreased permeability. Not only all cAMP-elevating bio-ligands we tested but also FSK, db-cAMP, and IBMX activated Rap1. Consistently, cAMP-dependent Rap1 activation upon stimulation by these ligands involved Epac in the regulation of barrier function. A previous report showed that Rap1 is phosphorylated by PKA in neutrophils and platelets, although the function of phosphorylated Rap1 has not been elucidated (37). So far, Epac is known to regulate several biological functions including integrin-dependent cell adhesion, insulin secretion, and calcium release through ryanodine-sensitive Ca^{2+} channels (reviewed in reference 5). In addition to these Epac-mediated functions, we show, for the first time, that Epac-Rap1 signaling is important for regulation of endothelial barrier function.

AJ assembly contributes to the regulation of barrier function. Rap1 is involved in the formation and maintenance of AJ constituted by cadherin (23, 41). Recently, it has been reported that homophilic ligation of E-cadherin induced Rap1 activation, which may be responsible for maturation of AJ (20). Consistently, suppression of endogenous Rap1 inhibits formation of E-cadherin-dependent cell adhesion (36), suggesting the critical role of Rap1 in the establishment of cadherin-based cell-cell contacts. Here, we demonstrate that Rap1 also acts downstream of cAMP-Epac to potentiate VE-cadherin-depen-

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**FIG. 10.** cAMP induces cortical actin rearrangement in a Rap1-dependent manner. (A) Monolayer-cultured HUVECs starved in 0.5% BSA-containing medium 199 for 3 h were stimulated with vehicle (top row), 10 μg/ml PGI2 (second row), 10 μM FSK (third row), and 0.2 mM 8-CPT-2'-O-Me-cAMP (8CPT) (bottom row) for 30 min. Fixed and permeabilized cells were stained with rhodamine-phalloidin (left column) and with anti-cortactin (center column). Rhodamine images to detect F-actin (red) and Alexa 488 images for cortactin visualized by Alexa 488-labeled secondary antibody (green) were obtained through a confocal microscope (BX50WI). Right panels show the merged images of rhodamine and Alexa 488 images. Bars, 20 μm. (B) HUVECs transfected with an EGFP-expressing vector (left) and pCXN2-Rap1GAPII-IRES-EGFP (right) were serum starved in 0.5% BSA-containing medium 199 for 3 h and stimulated with vehicle (top panels) and 10 μM FSK (bottom panels). Cells were fixed, permeabilized, and stained with Rhodamine-phalloidin. EGFP images (green) and rhodamine images showing F-actin (red) were obtained similar to those in panel A. Arrows and arrowhead indicate transfected and untransfected cells, respectively. Bars, 20 μm. (C) Cell permeability of HUVECs pretreated with 2 μM cytochalasin D (CytoD) for 30 min followed by 10 μM FSK stimulation for 30 min was analyzed as described in the legend to Fig. 1A. −, absent; +, present. (D) The effect of pretreatment of 2 μM cytochalasin D (CytoD) on adhesion of HUVECs stimulated with FSK was analyzed as described in the legend to Fig. 5E. A significant difference between two groups determined by Student’s t test is indicated by double asterisks (P < 0.01).
dent cell adhesion, thereby improving barrier function. In addition to cAMP-elevating ligands, S1P, which enhances AJ formation and barrier function (18, 26), also activated Rap1 (our unpublished data). Thus, Rap1 may play a crucial role in barrier function induced by various types of barrier-improving factors.

Our data and previous studies show that cAMP protects thrombin-induced endothelial barrier dysfunction. cAMP does not limit the effect of thrombin on the initial loss of endothelial barrier (32). Instead, cAMP enhances the restoration of barrier function disrupted by thrombin. Recently, it was also reported that Cdc42 regulates the restoration of endothelial barrier function disrupted by thrombin (24). Thus, cAMP-Epac-Rap1 signaling may facilitate the formation of VE-cadherin-based cell-cell contacts, cooperatively or in parallel with Cdc42.

Rap1 enhances integrin-dependent cell adhesion in a variety of hematopoietic cells by modulating the affinity and avidity of integrin (6, 22). Cell adhesion to VEC-Fc-coated dishes was augmented by Rap1 activation, suggesting that the homophilic binding of VE-cadherin is also likely ascribed to the affinity and avidity of VE-cadherin modulated by Rap1-triggered inside-out signaling. Hogan et al. reported that Rap1 activity is required for the targeting of E-cadherin molecules into nascent cell-cell contact sites, which in turn leads to the maturation of E-cadherin-based cell-cell contacts (20). Thus, cAMP-Epac-Rap1 signaling may also regulate the recruitment of VE-cadherin into maturing cell-cell contacts. Since downstream signaling of Rap1 that increases homophilic binding of VE-cadherin has not yet been characterized, the effector of cAMP-Epac-Rap1 signaling will need to be identified.

The actin cytoskeleton is a critical determinant of vascular integrity (10). PGI2, FSK, and S-CPT-2’-O-Me-cAMP induced cortical actin rearrangement in a Rap1-dependent manner. FSK-induced VE-cadherin-dependent cell adhesion was inhibited by cytochalasin D. Thus, Rap1 may promote VE-cadherin-dependent cell adhesion by inducing cortical actin rearrangement. AF-6 may act downstream of Rap1 to regulate the actin cytoskeleton, since it binds to GTP-bound Rap1 and the actin cytoskeleton regulator, profilin, and is localized at AJ (2). Consistently, Canoe, the drosophila homolog of AF-6, and Rap1 function in the same molecular pathway during embryonic dorsal closure, which requires cell-cell contacts (3). S1P promotes endothelial barrier function by inducing Rac-dependent cortical actin rearrangement. S1P also induces Rap1 activation (our unpublished data). A previous report indicates that Rac can function downstream of Rap1 in the processing of the amyloid precursor protein (28). Taken together, Rac may act downstream of Rap1 to induce cortical actin rearrangement.

In conclusion, we have demonstrated that the cAMP-Epac-Rap1 signaling pathway promotes VE-cadherin-mediated cell adhesion and consequently improves endothelial barrier function.

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REFERENCES


21. Kinbara, K., L. E. Goldfinger, M. Hansen, F. L. Chou, and M. H. Ginsberg,


