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Sac-1004, a novel vascular leakage blocker, enhances endothelial barrier through the cAMP/Rac/cortactin pathway

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ABSTRACT

The maintenance of endothelial barrier is critical for the vascular homeostasis and is maintained by the interaction of adherens junction (AJ) and tight junction (TJ) proteins between adjacent cells. This interaction is stabilized by actin cytoskeleton forming cortical actin ring. Here, we developed a novel vascular leakage blocker, Sac-1004 and investigated its mechanism of action in endothelial cells (ECs). Sac-1004 inhibited endothelial hyperpermeability induced by vascular endothelial growth factor, histamine and thrombin via stabilization of cortical actin ring and AJ proteins at the cell–cell junction. Treatment of Sac-1004 in ECs increased cAMP levels and activated Rac, both of which are known to strengthen endothelial barrier. Furthermore, Sac-1004 induced phosphorylation of cortactin and its localization at cell membrane that is essential for the stabilization of cortical actin ring. These effects of Sac-1004 on ECs were significantly abrogated by dideoxyadenosine (adenylyl cyclase inhibitor) and NSC23766 (Rac inhibitor). Taken together, our findings indicate that Sac-1004 blocks vascular leakage by enhancing endothelial integrity via the cAMP/Rac/cortactin pathway and imply the potential usefulness of Sac-1004 in the development of therapeutic means for vascular leakage-related diseases.

1. Introduction

The network of blood vessel in the vascular system permits the blood flow throughout the body. The endothelial cell (EC) barrier lining the blood vessel controls vascular homeostasis by monitoring the exchange of fluid, macromolecules, nutrients, and leukocytes between the blood and the interstitial space [1,2]. Dysfunction of inter-EC contacts leads to the increased permeability followed by edema and inflammation as observed in several diseases, such as diabetic retinopathy, acute lung injury, ischemic stroke, cerebral infarction, and atherosclerosis. However, the pharmacological remedy of the diseases that occurs by the disruption of the endothelial barrier has not been developed [3–5]. Barrier integrity is strongly coordinated based on contractile forces produced by the actomyosin complex and interaction of actin cytoskeleton networks with cell junction proteins. Actin filaments are organized at the cell periphery to form cortical actin rings and, in turn, stabilize the AJs (consisting of VE-cadherin, α-, β-, γ-, and p120-catenins) and TJs (consisting of occludin, zonal occludens: ZO-1, ZO-2, claudins, and junctional adhesion molecules), resulting in the maintenance of endothelial barrier function [6,7].

Various permeability-inducing agents, such as vascular endothelial growth factor (VEGF), histamine, bradykinin, and thrombin, produce their function by destabilizing AJs and TJs. It has been well established that increases in cAMP levels prevent endothelial barrier permeability [8–10]. Rac, one of the members of Rho family of GTPases and a downstream effector of cAMP, maintains endothelial barrier function by promoting AJ assembly and its cooperation with actin cytoskeleton [10–12]. Several studies have shown that molecules such as atrial natriuretic peptide [13], prostaglandins E2 and I2 (PGE2 and PGI2) [8], WS® 1442 [14] and, OxPAPC [15] are known to increase cAMP levels and activate Rac, leading to the enhanced endothelial barrier functions. Accordingly, VEGF, histamine, bradykinin, and thrombin induce hyperpermeability by disrupting intercellular junctions and inducing actin stress fiber formation [1,4,16].

Recently, we demonstrated that Sac-0601, a pseudo-sugar derivative of cholesterol, blocked VEGF-induced endothelial permeability [17]. Subsequent screening of a series of derivatives led

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us to the discovery of Sac-1004. Herein, we examine the vascular leakage-preventing efficacy of Sac-1004 and investigate the mechanism underlying this activity.

2. Materials and methods

2.1. Drug

Sac-1004 (Leakage Blocker 1004) was synthesized as follows. Briefly, pregnenolone was reacted with dihydropyran in the presence of *p*-Toluenesulfonic acid to prepare tertrahydropyran-analog. After Wittig olefination of it using 4-(carboxybutyl)triphenylphosphonium bromide, acid moiety was methylated by trimethylsilyldiazomethane. Sac-1004 was synthesized via tetrahydropyran deprotection and subsequent glycosidation with 4,6-*di*-*O*-acetyl-2,3-dideoxyhex-2-enopyran in the presence of acid.

2.2. Cell culture and reagent

Human Umbilical Vein ECs (HUVECs) were isolated from human umbilical cord veins by collagenase treatment. Cells were grown in 2% gelatin coated dishes and maintained in M199 medium (Invitrogen) supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin, 3 ng/ml basic fibroblast growth factor (R&D Systems), and 5 U/ml heparin (Sigma) at 37 °C in a humidified 95–5% (v/v) mixture of air and CO_2. Human retinal ECs (HRECs) were purchased from Applied Cell Biology Research Institute (Kirkland,Wash.) and passages 2–7 were used for experiments. Cells were grown in 2% gelatin-coated dishes and maintained in EC basal medium (EBM-2, CC-3156) containing EGM-2-kit (CC-4176) (Clonetics,Lonza Walkersville) and 20% fetal bovine serum. Dideoxyadenosine and NSC23766 were purchased from Santa Cruz biotechnology and Calbiochem respectively.

2.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

HUVECs were seeded at a density of 3×10^4 cells/well in gelatin-coated 24-well plates and incubated overnight. Cells were washed and switched to serum free media and treated with various concentrations of Sac-1004. After 48 h cells were washed and serum free media containing MTT (0.1 mg/ml) was added followed by incubation at 37 °C for 3 h. The residual MTT was carefully removed and the crystals were dissolved by incubation with DMSO:Ethanol (1:1). The absorbance was measured at 560 nm spectrophotometry.

2.4. FITC-dextran permeability assay

HUVECs were grown until confluent onto gelatin coated transwell filter (Corning Costar). Cells were serum-starved in endothelial serum free medium for 3 h and treated with Sac-1004 (10 μg/ml) for 60 min before induction with VEGF (Upstate Biotechnology), thrombin (Sigma), and histamine (Sigma). FITC-dextran (1 mg/ml; Sigma) was added to the upper compartment. Absorbance from the lower chamber solution was measured at 492 nm excitation and 520 nm emission in a FLUOstar omega.

2.5. Immunofluorescence

HUVECs and HRECs were subjected to immunostaining as described previously [17]. Briefly, the cells were fixed in 3.7% formaldehyde for 20 min at room temperature. After fixation, the cells were permeabilized in 0.1% Triton X-100 in PBS for 15 min at 4 °C. Cells were incubated for overnight at 4 °C with antibodies such as anti-VE-cadherin, β-catenin, p-120 catenin (Santa Cruz Biotechnology), ZO-1, ZO-2 (Invitrogen). The cells were incubated with secondary antibody conjugated with Alexa for 1 h at room temperature. Actin filaments were monitored by rhodamine phalloidin (Molecular Probes) for 30 min. Cells were mounted using DAKO mounting reagent and were observed using a fluorescence microscope (Zeiss; 400×).

2.6. Rac pull-down assay

HUVECs grown in 100-mm dishes were incubated with Sac-1004 (10 μg/ml) for various time periods in serum-free media. Cells were lysed in lysis buffer and homogenized by passing through 26-gage needle. After centrifugation, supernatants were incubated with human p21-binding PAK-1 domain (residues 67–150), conjugated to agarose beads (10 μg, Upstate Biotechnology Inc.). The agarose beads were washed with the lysis buffer and resuspended in 2× SDS gel loading buffer. The samples were subjected to electrophoresis in 15% polyacrylamide gel, and Rac protein was detected using anti-Rac mouse monoclonal antibody.

2.7. Immunoprecipitation

Confluent HUVECs were rinsed with phosphate-buffered saline (PBS) and lysed. Lysates were passed several times through a 26-gage needle, and centrifuged for 15 min (14,000 rpm). Supernatant was collected and was incubated at 4 °C for 2 h with anti phosphotyrosine (PY-20) antibody (BD Biosciences). Protein A-agarose beads (20 μl) were added and then rotated at 4 °C for 60 min. The complex was washed prior to the addition of Laemmli sample buffer, boiled, and subsequent analyzed by electrophoresis. Western blotting was performed using monoclonal cortactin antibody (Upstate Biotechnology) and horseradish peroxidase-conjugated secondary antibody prior to visualization using enhanced chemiluminescence (Amersham Biosciences).

2.8. Western blot

HUVECs were washed with ice cold PBS and lysed in 200 μl RIPA buffer. Lysates were centrifuged at 14,000 rpm for 15 min. Protein samples were separated by electrophoresis on sodium dodecyl sulfate–polyacrylamide gel and transferred. Immunoblotting was performed with anti-VE-cadherin, -β-actin (Santa Cruz Biotechnology), and -Na,K-ATPase (Cell signaling technology) antibodies.

3. Results

3.1. Sac-1004 prevents disruption of endothelial barrier induced by permeability factors

Previous studies have shown that treatment of ECs with permeability factors, such as VEGF, thrombin, and histamine, break the prominent pattern of AJs and TJs and induce stress fiber formation, leading to gap formation [1,17,18]. To develop a drug that inhibits endothelial permeability, we synthesized pseudo-sugar derivative of cholesterol and named it Leakage Blocker Sac-1004 (referred to as 1004 in figures) (Fig. 1A). We found that Sac-1004 was able to increase EC viability during serum-starved condition (Fig. 1B). We evaluated the protective effects of Sac-1004 on endothelial barrier integrity. Treatment with Sac-1004 inhibited VEGF-induced FITC-dextran leakage in a transwell permeability assay, demonstrating preserved barrier function (Fig. 1C). To further clarify the role of Sac-1004 in the maintenance of vascular barrier integrity, we performed similar experiments using two more permeability factors: thrombin and histamine. Sac-1004 inhibited both

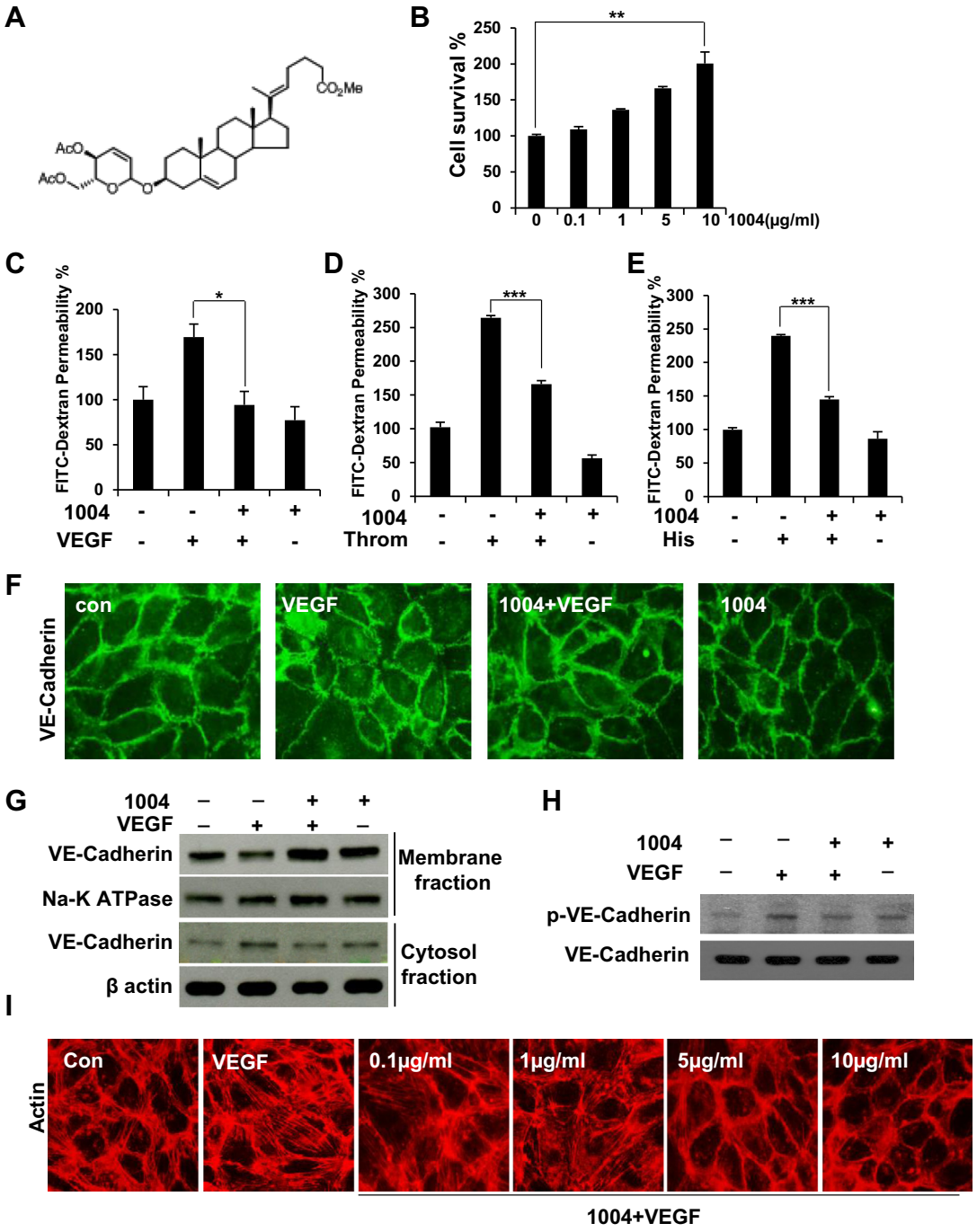

Fig. 1. Sac-1004 blocks endothelial hyperpermeability and stabilizes junctional proteins. Structure of Sac-1004 (A). Cells were treated with various concentration of Sac-1004. Cell survival was detected using MTT assay (B). HUVECs were treated with Sac-1004 (10 μg/ml, 60 min) followed by stimulation with VEGF (50 ng/ml, 60 min) (C), thrombin (throm, 2 U/ml, 15 min) (D), and histamine (his, 1 μM, 15 min) (E). Data are mean ± S.Ds; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. An *in vitro* transwell permeability assay was performed, and the amount of FITC-dextran diffused to the lower chamber was measured. HUVECs were treated with Sac-1004 (10 μg/ml, 60 min) followed by treatment with VEGF (50 ng/ml, 60 min). Cells were fixed, permeabilized, and subsequently stained with anti-VE-cadherin antibody (F). Cells were treated with Sac-1004 (10 μg/ml, 60 min) followed by stimulation with VEGF (50 ng/ml). Translocation of VE-cadherin protein to the membrane and cytosol fractions was observed using anti-VE-cadherin antibody (G). An immunoblot of phospho-Tyr^{685}-VE-cadherin was performed (H). Cells were treated with various concentrations of Sac-1004 followed by stimulation with VEGF. Cells were then fixed, permeabilized, and subsequently stained rhodamine phalloidin for F-actin (I).

thrombin- and histamine-induced FITC-dextran leakage (Fig. 1D and E). Furthermore, Sac-1004 also inhibited the disruptive effect of VEGF by restoring the linear distribution of the VE-cadherin (Fig. 1F), β-catenin, and p120-catenin (Supplementary Fig. 1A and B). We also observed that Sac-1004 was able to protect the VE-cadherin junction from the deleterious effect of thrombin and histamine (Supplementary Fig. 2A). Membrane fractionation and western blot revealed that the reduction in VE-cadherin levels in membrane fractions induced by VEGF was prevented by Sac-1004 (Fig. 1G). Moreover, VEGF-induced phosphorylation of Tyr^{685} of VE-cadherin, a marker of VE- cadherin disruption, was decreased by Sac-1004 (Fig. 1H). To illustrate the effect of Sac-1004 in ECs other than HUVECs, we examined the localization of TJ proteins in HREC. We found that VEGF-induced disruption of TJ

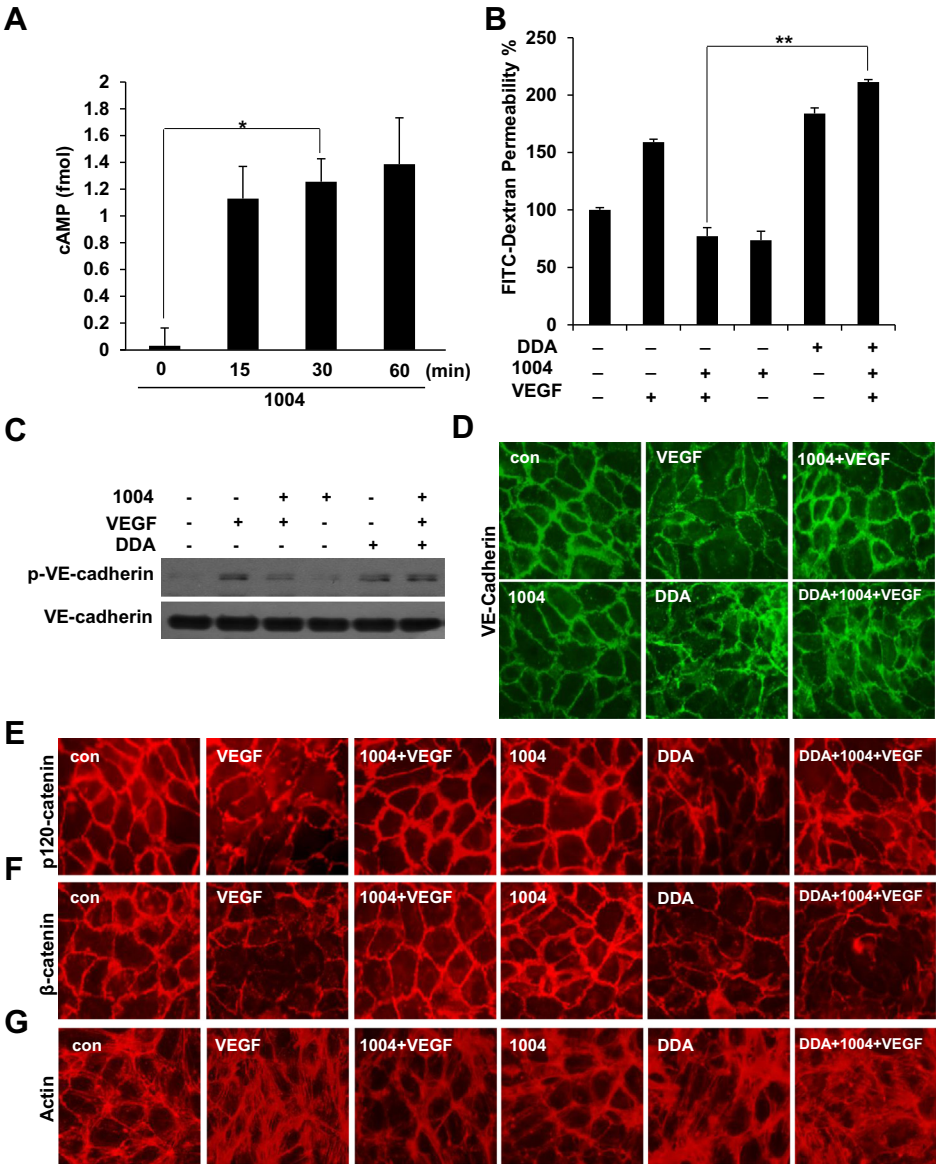

Fig. 2. cAMP pathway mediates Sac-1004-induced endothelial junction stabilizations. Cells were serum-starved for 3 h and treated with Sac-1004 (10 μg/ml) in time-dependent manner. Levels of cAMP were measured by ELISA (A). Confluent cells were serum-starved and pretreated for 30 min with dideoxyadenosine (DDA), followed by Sac-1004 (10 μg/ml, 60 min) treatment and VEGF (50 ng/ml, 60 min) treatment. An *in vitro* transwell permeability assay was performed, and the amount of FITC-dextran that diffused to the lower chamber was measured (B). Data are mean ± S.D; $^{**}P < 0.01$. Immunoblotting was done using anti-phospho-Tyr^{685}-VE-cadherin antibody (C). Immunofluorescence staining of cells was done using anti-VE-cadherin antibody (D), anti-p120-catenin antibody (E), anti-β-catenin antibody (F), and rhodamine phalloidin for F-actin (G).

proteins such as ZO-1 and ZO-2, at the HREC-membrane was also blocked by Sac-1004 (Supplementary Fig. 1C and D). Additionally, Sac-1004 prevented the VEGF-mediated stress fiber formation and maintained the cortical actin ring in a dose-dependent manner (Fig. 1I). Similarly, thrombin- and histamine-induced actin stress fiber formation was also blocked by Sac-1004 (Supplementary Fig. 2B). Collectively, these data show that Sac-1004 provides protection of endothelial barrier integrity against the permeability factors.

3.2. Induction of cAMP is essential for Sac-1004–induced protection of endothelial barrier

Increased cAMP levels have been known to enhance the endothelial barrier integrity [8–10]. To examine whether Sac-1004 exerts its effect through cAMP, we measured the cAMP levels in Sac-1004-treated cells. We found that Sac-1004 increased endothelial cAMP levels in a time-dependent manner (Fig. 2A). To confirm the role of cAMP in Sac-1004-induced endothelial barrier protection, we performed transwell permeability assay using dideoxyadenosine (DDA), an adenylyl cyclase inhibitor. Sac-1004 efficiently blocked VEGF-induced FITC-dextran diffusion. However, pretreatment of ECs with DDA prevented Sac-1004 from blocking the VEGF-induced permeability (Fig. 2B). Treatment with Sac-1004 did not decrease the VEGF-induced phosphorylation of Tyr^{685} in the presence of DDA (Fig. 2C). Furthermore, in the presence of DDA, Sac-1004 was unable to prevent the VEGF-induced disruption of AJ proteins VE-cadherin, p120-catenin and β-catenin (Fig. 2D–F) and cortical actin ring formation (Fig. 2G). Treatment with DDA also nullified the effect of Sac-1004 on thrombin- and histamine-induced leakage of FITC-dextran (Supplementary Fig. 3A and B), disruption of VE-cadherin and β-catenin at the cell

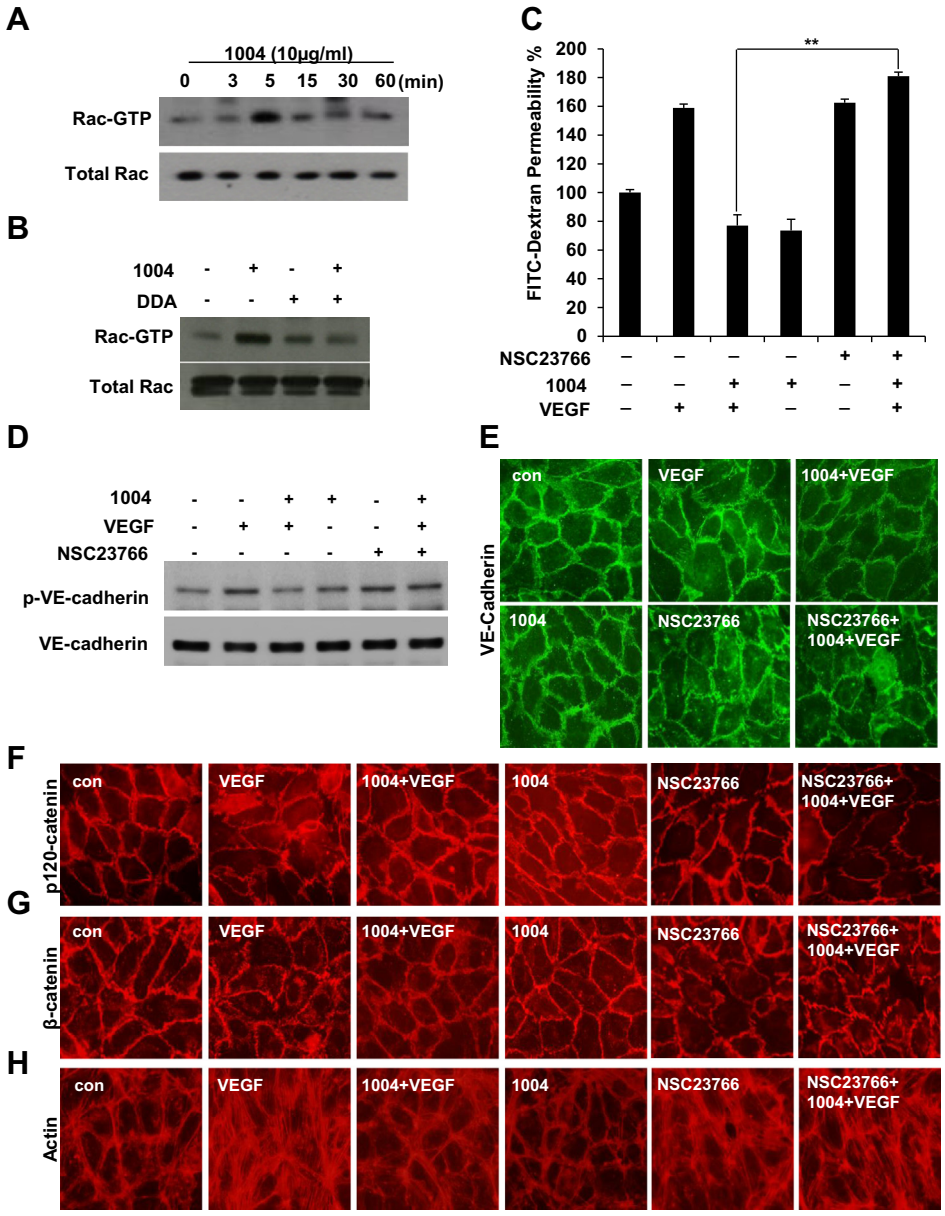

Fig. 3. Activation of Rac is involved in Sac-1004-induced endothelial junction stabilization. Confluent cells were serum-starved for 3 h and treated with Sac-1004 (10 μg/ml) in a time dependent manner. Rac-GTPase activity was assayed by using Rac Activation Assay kit (A). Confluent cells were serum-starved and pretreated with DDA, for 30 min, followed by Sac-1004 (10 μg/ml) treatment for 5 min. Rac-GTPase activity was measured (B). Confluent cells were serum-starved and pretreated with NSC23766, for 30 min, followed by Sac-1004 (10 μg/ml) treatment for 60 min and VEGF treatment (50 ng/ml, 60 min). An in *vitro* transwell permeability assay was performed, and the amount of FITC-dextran that diffused to the lower chamber was measured (C). Data are mean ± S.D; $^{*}P < 0.05$. Immunoblotting was done using anti–phospho-Tyr^{685}-VE-cadherin antibody (D). Immunofluorescence staining of cells was done using anti-VE-cadherin antibody (E), anti-p120-catenin antibody (F), anti-β-catenin antibody (G), and rhodamine phalloidin for F-actin (H).

membrane, and actin stress fiber formation (Supplementary Fig. 3C–E). Taken together, the Sac-1004-induced increase in cAMP is necessary for the inhibition of vascular permeability.

3.3. *Rac activation mediates the protective effect of Sac-1004 against endothelial permeability factors*

Elevated cAMP levels lead to the activation of Rac, a downstream element of cAMP signaling as well as a regulator of the actin cytoskeleton. Increased Rac activity has also been linked to strengthening of the vascular barrier [19,10,11]. To evaluate the role of Rac in Sac-1004–mediated protection of endothelial barrier function, we investigated Rac activity in Sac-1004–treated cells. Rac was induced 5 min after Sac-1004 treatment (Fig. 3A). Pretreatment with DDA inhibited the Sac-1004–induced activation of Rac (Fig. 3B). Similarly, transwell permeability assay demonstrated that Sac-1004 was unable to prevent the VEGF-mediated diffusion of FITC-dextran in the presence of NSC23766, a Rac inhibitor (Fig. 3C). Furthermore, treatment with Sac-1004 did not decrease the VEGF–induced phosphorylation of Tyr^{685} in the presence of NSC23766 (Fig. 3D). Similarly, Sac-1004 did not prevent the VEGF-induced disruption of VE-cadherin, p120-catenin and β-catenin (Fig. 3E–G) and actin stress fiber formation (Fig. 3H), in the presence of NSC23766. Treatment of the cells with NSC23766 also blocked the protective effect of Sac-1004 on thrombin- and histamine-induced permeability (Supplementary Fig. 3A and B), VE-cadherin and β-catenin disruption, and actin stress fiber formation (Supplementary Fig. 3C–E). These results demonstrate

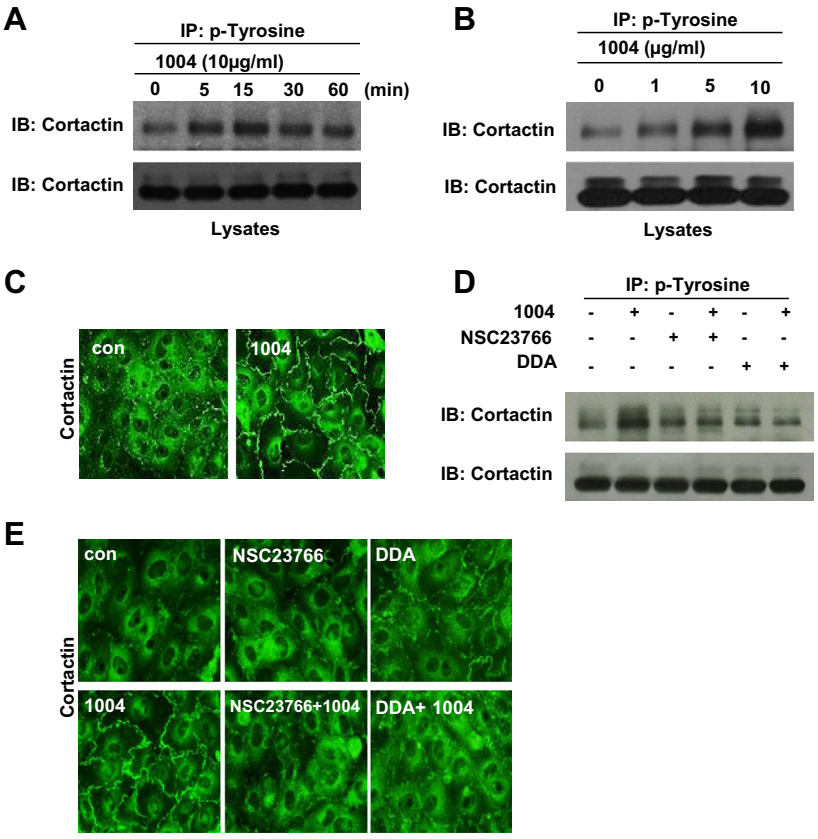

Fig. 4. Sac-1004 induces phosphorylation and localization of cortactin via the cAMP and Rac pathway. Confluent HUVECs were treated with or without Sac-1004 in time- (A) and dose- (B) dependent manner. Cells were lysed, and proteins were immunoprecipitated with anti-phosphotyrosine (PY-20) antibody. These were used to immunoblot for cortactin. Confluent HUVECs were pretreated with or without Sac-1004 (10 μg/ml) and stained with anti- cortactin antibody (C). Cells were serum-starved and pretreated with DDA, NSC23766 for 30 min followed by treatment with Sac-1004 (10 μg/ml, 15 min). A western blot was performed using anti-cortactin antibody (D). Immunofluorescence staining of cells was done using anti-cortactin antibody (E).

that Sac-1004 activates Rac via cAMP to antagonize the effect of permeability factors in ECs.

3.4. Cortactin contributes to endothelial barrier protection by Sac-1004

Cortactin, a downstream effector of Rac and an actin-binding protein, is phosphorylated and localized at the cell membrane, strengthening the cortical actin ring [10,19,20]. Immunoprecipitation with a phospho-tyrosine antibody revealed that Sac-1004 treatment activated cortactin by phosphorylation at tyrosine residue in time- and dose-dependent manner (Fig. 4A and B). Furthermore, immunofluorescence staining of cortactin also illustrated that Sac-1004-mediated the localization of cortactin at the cell membrane (Fig. 4C). Importantly, Sac-1004-induced activation of cortactin was reduced with DDA and NSC23766 treatments (Fig. 4D). Similarly, the localization of cortactin to the membrane induced by Sac-1004 also diminished upon DDA and NSC23766 treatment (Fig. 4E). These results demonstrate that Sac-1004 stabilizes cortical actin ring by activation of cortactin via cAMP and Rac.

4. Discussion

Vascular endothelial hyperpermeability is hallmark of severe pathological conditions like diabetic retinopathy, ischaemia, brain stroke, cancer and inflammation. In this study, we developed a vascular leakage blocker on the basis of cell based assay which is capable of protecting the endothelial barrier integrity. We also analyzed the various aspects of endothelial barrier enhancement and demonstrated that cAMP/ Rac/cortactin pathway is responsible for the protection of endothelial barrier integrity induced by Sac-1004.

The cytoplasmic tail of VE-cadherin binds other AJ proteins such as β-catenin and p120-catenin, which in turn interact with actin cytoskeleton and zonula occludens [12]. This association is necessary for the regulation of endothelial permeability and junction stabilization. Internalization of VE-cadherin is associated with junction deterioration [21,22]. Several studies have shown that treatment with permeability factors results in dissolution of AJ proteins [18,23–25]. We also observed the VEGF-induced internalization of VE-cadherin from membrane to cytosol. Immunofluorescence showed that the membrane localization of AJ proteins: VE-cadherin, β-catenin and p120-catenin, was disrupted by VEGF, thrombin or histamine treatment, contributing to the endothelial permeability. Treatment with Sac-1004 stabilized the AJ proteins at the cell membrane. AJs have been known to contribute in strengthening of the endothelial barrier function, such as in the blood–brain barrier and blood-retinal barrier [3,26,27]. Strengthening of blood-retinal barrier is also mediated by TJ proteins in neighboring retinal ECs. Sac-1004 treatment also blocked the VEGF-induced breakage of the TJ proteins ZO-1 and ZO-2 by maintaining its linear pattern in the HRECs. Thus, Sac-1004 not only protects endothelial barrier in peripheral blood vessels but also in the retinal blood vessels.

Actin filaments at cell periphery form a cortical actin ring that, together with AJs and TJs, strengthens the endothelial barrier. Dissolution of the cortical actin ring by permeability factors leads to actin stress fiber formation and destabilizes the barrier integrity

[10,17,28,29]. In this study, we demonstrated that Sac-1004 antagonizes VEGF-, thrombin- and histamine-induced actin stress fiber formation and stabilizes the cortical actin ring. In quiescent cells, cortactin, the actin binding protein, assembles at the cell circumference. Localization of cortactin to cell edges is essential for the endothelial barrier enhancement [10,19,20]. We clearly demonstrated Sac-1004-induced cortactin phosphorylation and localization to membrane. When adenylyl cyclase and Rac were inhibited, Sac-1004 was unable to induce phosphorylation and membrane localization of cortactin. These results are consistent with the previous findings that cAMP and Rac activate cortactin and reduce endothelial permeability. Higher levels of cAMP have been shown to activate Rac via Protein Kinase A (PKA) and EPAC (exchange protein directly activated by cAMP)-Rap1 pathways. Augmented cAMP levels and Rac activation have been linked to reduced endothelial permeability [10,11,19,20]. Previous studies have shown that increased intracellular cAMP levels can block the permeability induced by thrombin [30], histamine [31] and VEGF [32]. Consistent with these findings, we showed that Sac-1004 can significantly increase cAMP levels and activate Rac. Adenylyl cyclase inhibitor (DDA) treatment blocked the activation of Rac by Sac-1004, demonstrating that Rac activation is cAMP-dependent.

VEGF is known to be involved in altering endothelial permeability in the diseases like diabetic retinopathy, ischaemia–reperfusion injury, and tumor development and metastasis [33]. Also, histamine causes microvascular leakage during the acute inflammatory response in clinical conditions like trauma, burns, allergy and some infectious disease [34]. Considering the involvement of VEGF and Histamine in a number of diseases, Sac-1004 has a promising implication in treatment of such vascular permeability-associated conditions. In support of this statement, we have demonstrated that Sac-1004 can efficiently prevent the VEGF-induced retinal vascular leakage and cure streptozotocin-induced leakage in diabetic retina (unpublished data). However, the receptor for Sac-1004 is not yet known. Since the activation of G-protein-coupled receptor (GPCR) is known to increase the cAMP level in the cells by activation of adenylyl cyclase, one of the GPCRs could be the possible receptor for Sac-1004.

In conclusion, these findings demonstrate that Sac-1004 prevents ECs permeability by maintaining cell junction stability. Mechanistically, we show that Sac-1004 elevates cAMP levels and activates Rac signaling, leading to cortactin phosphorylation and endothelial barrier enhancement. Thus, Sac-1004 holds promise as a potential therapeutic agent for several diseases associated with vascular leakage.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.104.

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