

Prostaglandin D₂-DP Signaling Promotes Endothelial Barrier Function via the cAMP/PKA/Tiam1/Rac1 Pathway

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Objective—Prostaglandin D₂ (PGD₂) is one of the prostanoids produced during inflammation. Although PGD₂ is known to decrease endothelial permeability through D prostanoid (DP) receptor stimulation, the detailed mechanism is unknown.

Methods and Results—Treatment with PGD₂ (0.1–3 μmol/L) or the DP receptor agonist, BW245C (0.1–3 μmol/L), dose-dependently increased transendothelial electrical resistance and decreased the FITC-dextran permeability of human umbilical vein endothelial cells. Both indicated decreased endothelial permeability. These phenomena were accompanied by Tiam1/Rac1-dependent cytoskeletal rearrangement. BW245C (0.3 μmol/L) increased the intracellular cAMP level and subsequent protein kinase A (PKA) activity. Pretreatment with PKA inhibitory peptide, but not gene depletion of exchange protein directly activated by cAMP 1 (Epac1), attenuated BW245C-induced Rac1 activation and transendothelial electric resistance increase. In vivo, application of 2.5% croton oil or histamine (100 μg) caused vascular leakage indexed by dye extravasation. Pretreatment with BW245C (1 mg/kg) attenuated the dye extravasation. Gene deficiency of DP abolished, or inhibition of PKA significantly reduced, the DP-mediated barrier enhancement.

Conclusion—PGD₂-DP signaling reduces vascular permeability both in vivo and in vitro. This phenomenon is mediated by cAMP/PKA/Tiam1-dependent Epac1-independent Rac1 activation and subsequent enhancement of adherens junction in endothelial cell. (*Arterioscler Thromb Vasc Biol.* 2013;33: 565-571.)

Key Words: endothelial permeability ■ prostaglandin D₂

Prostaglandins (PGs), metabolites of cyclooxygenase, participate in several inflammatory responses, including modulation of vascular permeability, platelet aggregation, fever, and pain generation. PGD₂ is an important PG abundantly produced by mast cells and exerts its biological function via 2 distinct receptors: the D prostanoid (DP) receptor and the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2).¹ PGD₂ is well recognized as a mediator of allergic inflammation,^{2,3} which stimulates recruitment of Th2 cells, eosinophils, and basophils. However, the effects of PGD₂ on vascular function are not well known. We previously focused on the effects of PGD₂ on vascular endothelial function and found that PGD₂ contributes to endothelial barrier enhancement in growing tumor through endothelial DP receptor stimulation.⁴ However, the details of this process remain unknown.

Endothelial cells lining the intima of blood vessels form a semipermeable barrier and regulate fluid and solute exchange between blood and the interstitium.⁵ This barrier is indispensable in maintaining tissue homeostasis. Its dysfunction is critical in the pathogenesis of inflammatory disease. In acute inflammation, including sepsis and allergy, proinflammatory mediators disrupt the endothelial barrier and induce tissue

edema and leukocyte extravasation.⁵ Under chronic inflammatory conditions as observed during tumor growth and rheumatism, persistent exposure mainly to vascular endothelial growth factor induces continuous leukocyte infiltration and subsequent angiogenesis which promote the pathology.^{6,7} Because endothelial barrier enhancement is likely to inhibit an excessive inflammatory response, discovering a novel barrier enhancer and elucidating its method of action may potentially provide new therapeutic applications for various types of disease.

There are 2 common signaling pathways that promote endothelial barrier function, the cAMP- and the PI3K-dependent pathways. Forskolin and isoproterenol are known to promote endothelial barrier function through cAMP-dependent activation of protein kinase A (PKA)⁸ or exchange protein directly activated by cAMP (Epac).⁹ In contrast, sphingosine-1-phosphate promotes endothelial barrier integrity through the PI3K-dependent pathway.^{10,11} Both cAMP- and PI3K-dependent signaling pathways converge on a Rho family of GTPase, Rac1. Activated Rac1 in turn induces cortical actin rim formation and subcellular vascular endothelial (VE)-cadherin redistribution from the cytoplasmic to the membrane fraction, which result in adherens junction formation and its stabilization.¹²

Received on: February 1, 2012; final version accepted on: December 26, 2012.

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Arterioscler Thromb Vasc Biol is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.112.300993

Here, we investigated the signaling pathway underlying PGD₂-mediated endothelial barrier enhancement and found that it is mediated via cAMP/PKA/Tiam1-dependent Rac1 activation.

Materials and Methods

Detailed protocols for some experiments are described in online-only Data Supplement.

Cell Culture and siRNA Transfection

Human umbilical vein endothelial cells (HUVECs; Lonza, Switzerland; passage 3–9) were used for the experiments after serum starvation for 4 hours. To deplete endogenous Tiam1, Trio, and Epac1 expression, HUVECs were transfected with 25 to 50 nmol/L gene-specific siRNA using lipofectamine RNAiMAX (0.2%). The cells were used 48 hours after transfection.

In Vitro Endothelial Permeability Assay

Endothelial barrier integrity was evaluated by measuring transendothelial electrical resistance (TER) using an Xcelligence Real-time Cell Analyzer DP system (Roche, Switzerland). The TER value at each time point was shown as a ratio to initial value.

In transwell permeability assay, cells were seeded in the upper chamber of transwell inserts (1 μm pore size, BD Falcon). After stimulation, 70 000 MW FITC-dextran (24 μg in 300 μL of medium) was added to the upper chamber. The amount of FITC-dextran infiltrating into the lower chamber was determined using a fluorescence spectrophotometer (PerkinElmer Japan, Japan).

Cell and En Face Immunostaining

Cells were fixed, permeabilized with 0.05% Triton X-100 for 15 minutes, and blocked with 3% BSA for 30 minutes. They were then incubated with goat anti-VE-cadherin antibody and labeled with secondary antibody and rhodamine-phalloidin. Nuclei were labeled with DAPI.

En face staining was performed as previously described.¹³ The external pulmonary artery was excised from mice. The arteries were fixed and permeabilized with 0.3% Triton X-100. They were incubated with goat anti-VE-cadherin antibody followed by secondary antibody labeling. Nuclei were labeled with DAPI. Images were obtained using a fluorescence microscope (Eclipse E800; Nikon, Japan).

Measurement of cAMP Accumulation

Cells were pretreated with a phosphodiesterases inhibitor, IBMX (200 μmol/L), for 5 minutes. After stimulation, cells were lysed and the cAMP concentration in the lysate was measured by enzyme immunoassay (cAMP Biotrak Enzyme immunoassay system; GE Healthcare, United Kingdom).

PKA Activity Measurement

PKA activity measurement was performed using a PepTag Non-Radioactive PKA Assay kit (Promega). Cells were stimulated with each reagent and lysed. PepTag A1 peptide was added to the lysates. Phosphorylated peptide was isolated by 0.8% agarose gel electrophoresis. The fluorescence bands were detected using an ultraviolet transilluminator (TOYOBO, Japan).

Rac1 and Cdc42 Activation Assay

The Rac1 and Cdc42 activation assay was performed using a Rac1/Cdc42 Activation Assay Kit (Millipore). HUVECs were stimulated and lysed. GTP-bound Rac1 and Cdc42 were captured using a pull-down assay with PAK-1 PBD agarose. The level of activated Rac1 or Cdc42 was measured by Western blot analysis and normalized to the total amount of Rac1 or Cdc42.

Western Blot Analysis

Cell lysates were electrophoresed on SDS-polyacrylamide gel and blotted to a polyvinylidene fluoride membrane. After blocking with 3% BSA, the membrane was probed with mouse anti-Rac1 antibody, rabbit anti-Cdc42 antibody, mouse anti-β-actin antibody, or rabbit anti-Epac1 antibody. Membrane was then labeled with secondary antibody conjugated to Alexa Fluor 680. Bands were detected with the Odyssey System (LI-COR Biosciences).

Mice

All experiments were approved by the institutional animal care and use committees of the University of Tokyo. DP-deficient mice (n=23) were generated, back-crossed >10 generations to C57BL/6CrSlc as described.² Wild-type mice (n=28) were used as controls.

Modified Miles Assay

Mice were anesthetized with isoflurane inhalation. Croton oil (2.5%) was applied or histamine (100 μg) was injected to the right auricle. Ninety minutes after croton oil or 10 minutes after histamine stimulation, Evans Blue (60 mg/kg) was administered intravenously and circulated for 30 minutes. The ears were excised, dried, and weighed. Evans Blue was extracted in formamide and its content was quantified at 610 nm in a spectrophotometer.

Intravital Microscopy

Mice were anesthetized with isoflurane inhalation and BW245C was intraperitoneally injected. Ten minutes later, FITC-dextran (2 mg/mL, 100 μL) was intravenously injected and croton oil (2.5%) was applied onto the auricle. FITC-dextran leakage and vascular diameter of arteriole were monitored for 30 minutes after croton oil stimulation using confocal microscope (Nikon, Japan).

Reagents

The following reagents were used: PGD₂, 13,14-dihydro-15-keto PGD₂, BW245C, BWA868C, and iloprost (Cayman chemical); IBMX and croton oil (Sigma-Aldrich); thrombin and PKA inhibitory peptide (Calbiochem, Germany); forskolin and histamine (Wako, Japan); NSC-23766 (Tocris Bioscience); O-Me-cAMP (Biolog, Germany); dextran fluorescein and rhodamine-phalloidin (Molecular Probes); lipofectamine RNAiMAX and Epac1 stealth Select 3 RNAi sets (Invitrogen); siGENOME SMARTpool against Tiam1 or Trio (Thermo); and goat anti-VE-cadherin antibody and rabbit anti-Epac antibody (Santa Cruz).

Statistical Analysis

The results of the experiments are expressed as means±SEM. Statistical evaluation of the data was performed by the unpaired Student *t* test for comparisons between 2 groups and by one-way ANOVA followed by the Bonferroni post-test for comparison between >2 groups. A value of *P*<0.05 was taken as significant.

Results

PGD₂ Promotes Endothelial Barrier Function via DP Receptor

We first assessed the effects of PGD₂ signaling on endothelial barrier function by measuring TER. As shown in Figure 1A and 1B, PGD₂ (0.1–3 μmol/L) increased TER in a dose-dependent manner, indicating enhanced endothelial barrier function. This response reached a maximum within 15 minutes and gradually declined over 2 hours. A DP receptor agonist, BW245C (0.1–3 μmol/L), but not a CRTH2 receptor agonist, DK-PGD₂ (0.1–1 μmol/L), dose-dependently increased TER (Figure 1C–1E). The same results were obtained

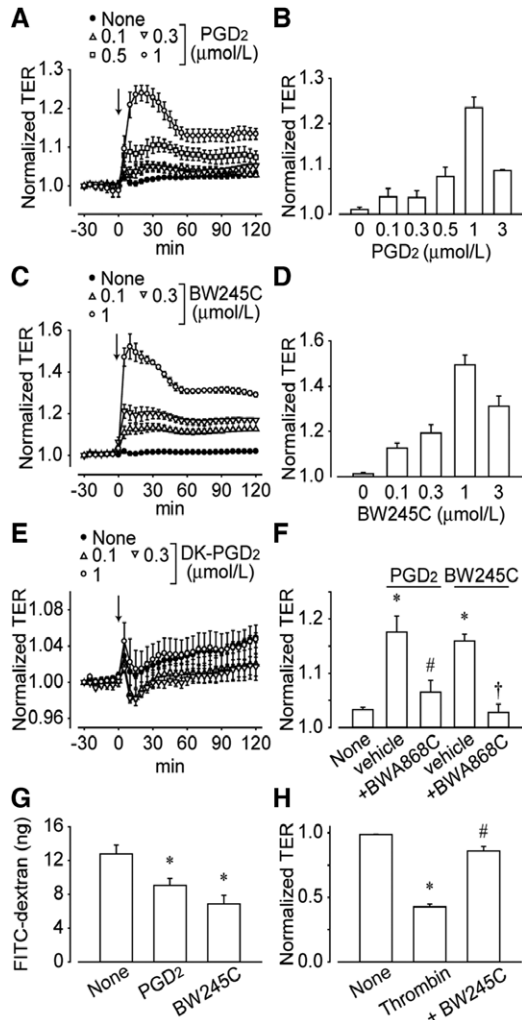


Figure 1. Prostaglandin D₂ (PGD₂)-DP signaling promotes endothelial barrier function. **A**, Effect of PGD₂ on transendothelial electrical resistance (TER; n=4–8). **B**, TER increase at 15 min after PGD₂ stimulation (n=4–8). **C**, Effect of BW245C on TER (n=4–6). **D**, TER increase at 15 min after BW245C stimulation (n=4–6). **E**, Effect of DK-PGD₂ on TER (n=4). **F**, Effect of pretreatment with BWA868C on PGD₂- and BW245C-induced TER increase (n=6–8). **P*<0.05 compared with none. #*P*<0.05 compared with PGD₂-treated cells. †*P*<0.05 compared with BW245C-treated cells. **G**, Effects of PGD₂ and BW245C on FITC-dextran permeability (n=5–6). **P*<0.05 compared with none. **H**, Effect of pretreatment with BW245C on thrombin-induced TER decrease (n=4–6). **P*<0.05 compared with none. #*P*<0.05 compared with thrombin-treated cells. Data are presented as mean±SEM. **Arrows** indicate the time points of stimulation.

in human dermal microvascular endothelial cells (Figure I in the online-only Data Supplement). Pretreatment with a DP receptor antagonist, BWA868C (30 μmol/L, 30 minutes), significantly inhibited both PGD₂ (1 μmol/L)- and BW245C (0.3 μmol/L)-induced TER increase to 42% and 18%, respectively, compared with the response when using each stimulant alone (Figure 1F, 15 minutes after the treatment). In dextran permeability assay, treatment with either PGD₂ (1 μmol/L) or BW245C (0.3 μmol/L) reduced permeability to 71% or 54% of the untreated controls, respectively (Figure 1G).

We next examined whether DP agonism is effective against barrier disruptive stimulation. Thrombin (0.1 U/mL) decreased TER

to 43% of untreated controls. Pretreatment with BW245C (0.3 μmol/L, 15 minutes) attenuated thrombin-induced TER decrease to 86% of untreated controls (Figure 1H, maximal response).

DP Agonism Induces Cytoskeletal Rearrangement and Adherens Junction Assembly Through Rac1 Activation

The effect of DP agonism on actin arrangement and adherens junction assembly in HUVECs was examined. As shown in Figure 2A, VE-cadherin was located at cell–cell contact areas under resting conditions (upper left panel). These unstimulated endothelial cells show actin stress fibers (arrowheads in the middle left panel), but did not exhibit a distinct cortical actin rim. Treatment with BW245C (0.3 μmol/L, 15 minutes) strongly enhanced VE-cadherin signaling at the cell border, reduced the number of actin stress fibers, and induced the formation of a cortical actin rim (arrows in middle panel), which indicated adherens junction assembly.

Because Rac1 is a critical modulator of actin rearrangement and adherens junction formation, we examined the involvement of Rac1 in DP-mediated endothelial barrier enhancement. Pretreatment with a Rac inhibitor, NSC-23766 (300 μmol/L, 1 hour), inhibited both the actin rearrangement and adherens junction assembly that were induced by BW245C (0.3 μmol/L, 15 minutes; Figure 2A, right panels). Pretreatment with NSC-23766 consistently abolished the BW245C (0.3 μmol/L)- and PGD₂ (1 μmol/L)-induced TER increase (Figure 2B and 2C, 15 minutes after the treatment).

Rac1 is activated by Rac-specific guanine nucleotide exchange factors, including Tiam1, Trio, and Vav2. Because NSC-23766 inhibit Rac1 activity by interfering Tiam1 and Trio,¹⁴ we examined the role of these 2 Rac-specific guanine nucleotide exchange factors in endothelial barrier formation. A siRNA transfection against Tiam1 attenuated DP agonism (BW245C, 0.3 μmol/L)-induced TER increase, whereas a siRNA transfection against Trio did not affect it (Figure 2D, 15 minutes after the treatment).

cAMP/PKA Signaling Is Required for DP-Mediated Endothelial Barrier Enhancement

As DP receptor coupling to the Gs subunit of G protein, we examined the role of cAMP and its effectors on BW245C-induced barrier enhancement. As expected, treatment of HUVECs with BW245C (1 μmol/L, 5–15 minutes) resulted in a 2.5-fold increase of the intracellular cAMP level in a time-dependent manner over untreated controls (Figure 3A). Treatment with forskolin (FSK; 1 μmol/L, 5 minutes), an adenylate cyclase activator, resulted in an even larger 6.6-fold increase.

An increase in the intracellular cAMP level is known to promote endothelial barrier integrity through the action of PKA or Epac1.⁹ As shown in Figure 3B, treatment with BW245C (0.3 μmol/L, 5–15 minutes) increased PKA activity in a time-dependent manner up to 1.3-fold over the control. This increase was reduced by pretreatment with cell-permeable PKA inhibitory peptide (PKI; 30 μmol/L, 1 hour). This pretreatment consistently attenuated the BW245C-induced TER

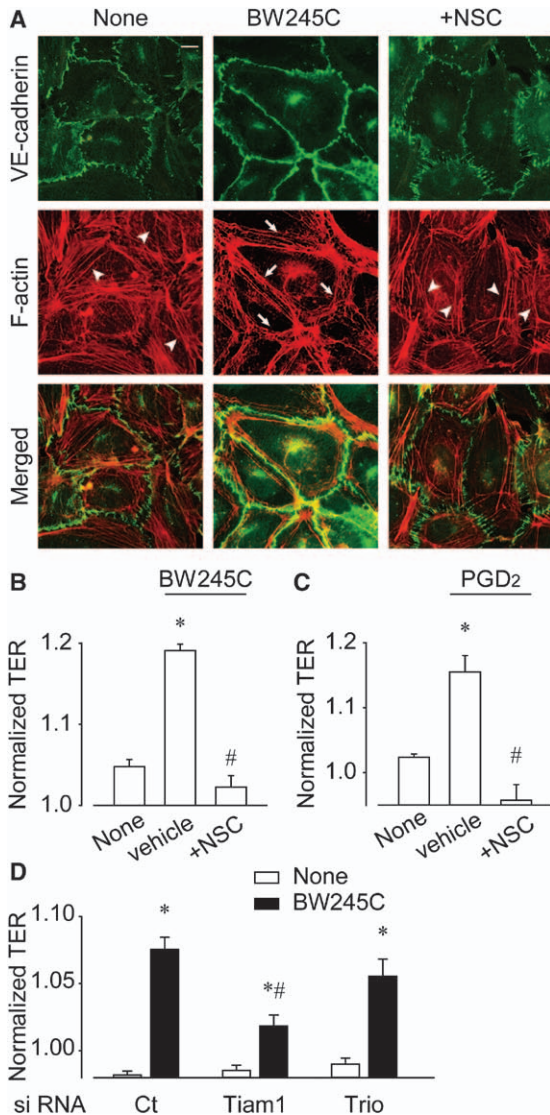


Figure 2. DP agonism induces cortical actin rim formation and adherens junction assembly in a Tiam1/Rac1-dependent manner. **A**, Typical pictures of immunostaining of vascular endothelial (VE)-cadherin (upper, green) and F-actin (middle, red) after BW245C stimulation with or without NSC-23766 pretreatment. **Bottom** shows merged pictures of VE-cadherin, F-actin, and DAPI (blue) staining. **Arrowheads** indicate stress fibers, **arrows** indicate cortical actin rim. Scale bar: 10 μ m. **B**, Effect of pretreatment with NSC-23766 on BW245C-induced transendothelial electrical resistance (TER) increase (n=8–9). * P <0.05 compared with none. # P <0.05 compared with BW245C-treated cells. **C**, Effect of pretreatment with NSC-23766 on prostaglandin D₂ (PGD₂)-induced increase TER (n=5–8). * P <0.05 compared with none. # P <0.05 compared with PGD₂-treated cells. **D**, Effect of Tiam1 and Trio depletion on BW245C-induced TER increase (n=4–8). * P <0.05 compared with none. # P <0.05 compared with control (ct) siRNA/BW245C-treated cells. Data are presented as mean \pm SEM.

increase to 62% of the control (Figure 3C, 15 minutes after the treatment).

We next assessed the contribution of Epac1 to DP-mediated endothelial barrier enhancement. In HUVECs, Epac1 siRNA transfection significantly reduced its protein expression to 25% of the control siRNA transfection (Figure 3D). This gene depletion of Epac1 impaired basal endothelial barrier function

to 64% of the control (data not shown), suggesting a role for Epac1 in maintaining resting barrier function. This gene depletion did not affect the BW245C-induced TER increase, whereas it did eliminate any TER response to a selective Epac activator, O-Me-cAMP (1 μ mol/L; Figure 3E, 15 minutes after the treatment).

Several studies suggested the contribution of other signaling factors, PI3K and Rho in endothelial barrier integrity. However, pretreatment with neither a PI3K inhibitor (LY294002, 10 μ mol/L, 1 hour) nor a Rho kinase inhibitor (Y-27632; 1 μ mol/L, 30 minutes) influenced BW245C (0.3 μ mol/L)-induced TER increase (Figure IIA and IIB in the online-only Data Supplement).

DP Agonism Induces Rac1 and Cdc42 Activation

Rac1 and Cdc42, members of Rho family GTPases enhance endothelial barrier through actin cytoskeleton rearrangement. We directly assessed these GTPase activities by pull-down assay for GTP-bound Rac1 or Cdc42. Treatment with BW245C (0.3 μ mol/L, 15 minutes) significantly increased the amount of GTP-Rac1 in HUVECs (Figure 4A). Pretreatment with either PKI (30 μ mol/L, 1 hour) or NSC-23766 (300 μ mol/L, 1 hour) abolished BW245C-induced Rac1 activation. Consistent with the TER measurement results, Epac1 gene depletion reduced Rac1 activity under resting conditions to 56% of control siRNA treatment, but did not affect the BW245C-induced elevation (Figure 4B). Treatment with BW245C (0.3 μ mol/L, 15 minutes) also induced a 2-fold increase in the amount of GTP-bound Cdc42 over the control (Figure 4C). Treatment with a PGI₂ analog, iloprost (1 μ mol/L, 15 minutes), activated Cdc42 as same level as BW245C.

DP Agonism Attenuates Endothelial Hyperpermeability In Vivo

We examined DP-mediated barrier enhancement in vivo mouse model. Croton oil was applied to the right ear of WT mice increasing dye extravasation (Figure 5A). An i.p. injection of BW245C (1 mg/kg, 10 minutes before Evans Blue administration) attenuated dye extravasation to 22% of control in the WT mice (Figure 5A and 5B). BW245C-treatment also attenuated histamine (100 μ g)-induced Evans Blue extravasation to 30% of control in WT mice (Figure 5C). When injected with PKI (0.5 mg/kg, intradermally, 1 hour before BW245C-treatment), croton oil-induced dye extravasation was reduced by only 64% of control in WT mice (Figure 5B).

DP-deficient (DP^{-/-}) mice showed equal vascular leakiness to WT mice without stimulation (WT: 0.187 \pm 0.01 mg/g, DP^{-/-}: 0.184 \pm 0.01 mg/g), but exhibited greater dye leakage with croton oil stimulation than WT mice suggesting a barrier enhancement role for endogenous PGD₂-DP signaling in vivo. As expected, DP^{-/-} mice showed almost no response to DP receptor stimulation with BW245C (Figure 5B).

We then examined the effect of DP agonism on endothelial adherens junctions by en face VE-cadherin immunostaining. As shown in Figure 5D, VE-cadherin was located at cell adhesion areas under resting conditions in WT mice (upper left panel). Treatment with BW245C (1 μ mol/L, 15 minutes) significantly increased VE-cadherin signal at the cell border,

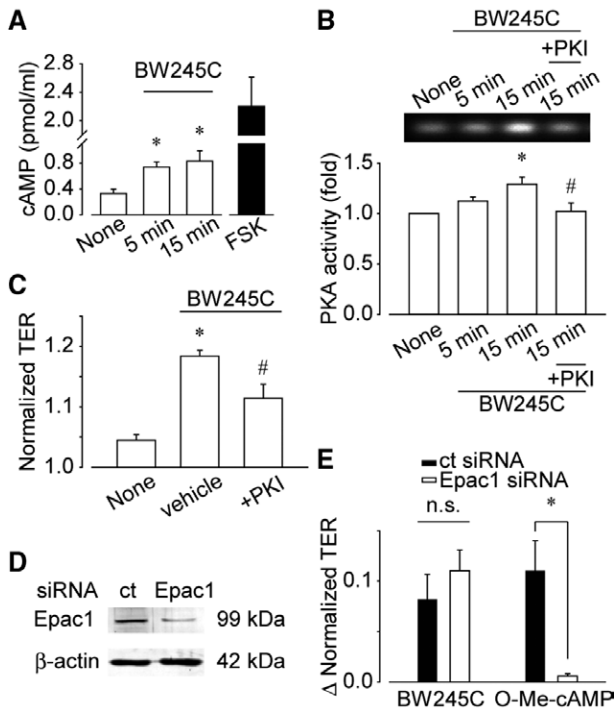


Figure 3. DP agonism promotes endothelial barrier through cAMP/protein kinase A (PKA) signaling pathway. **A**, Effect of BW245C or forskolin (FSK) on intracellular cAMP level (n=6). **P*<0.05 compared with none. **B**, Effect of BW245C on PKA activity with or without PKA inhibitory peptide (PKI) pretreatment (n=4). **Top** shows typical picture of phosphorylated PKA substrates and **bottom** shows the summary. Relative activity was calculated as folds of none. **P*<0.05 compared with none. #*P*<0.05 compared with 15 min-BW245C-treated cells. **C**, Effect of pretreatment with PKI on BW245C-induced transendothelial electrical resistance (TER) increase (n=9–11). **P*<0.05 compared with none. #*P*<0.05 compared with BW245C-treated cells. **D**, Epac1 and β-actin protein expression level after control (ct) or Epac1 siRNA transfection. **E**, Effect of Epac1 depletion on BW245C- or O-Me-cAMP-induced TER increase (n=4). TER increments were indicated as Δ normalized TER. **P*<0.05 compared with ct siRNA-treated cells. n.s. indicates not significant. Data are presented as mean±SEM.

indicating enhancement of adherens junction formation (upper right panel). Treatment with thrombin (0.1 U/mL, 15 minutes) disassembled VE-cadherin (lower left panel), which

was attenuated by pretreatment with BW245C (1 μmol/L, 15 minutes; lower right panel). DP^{-/-} mice, although endothelial cell arrangement seems to be normal at resting condition (Figure 5E, upper left panel), treatment with BW245C (1 μmol/L, 15 minutes) could neither assemble VE-cadherin to cell adhesion area nor counteract the action of thrombin (0.1 U/mL, 15 minutes; Figure 5E, upper right and lower right).

The tissue blood flow as well as endothelial barrier influence vascular permeability by modulating intravascular hydrostatic pressure. As shown in Figure 6, croton oil stimulation caused dextran leakage, at the same time it induced rapid and continuous vasodilation within 10 minutes suggesting a blood flow elevation. DP agonism (BW245C; 1 mg/kg, i.p., 10 minutes before the oil treatment) reduced the oil-induced hyperpermeability. However, DP agonism did not influence the vasodilative response to the oil stimulation. DP deficiency slightly but not significantly increased dextran leakage (Figure 6A and 6B), whereas it did not affect vasodilative response (data not shown). As expected, DP^{-/-} mice did not respond to DP agonism.

Discussion

Prostaglandins are known to influence endothelial barrier function and regulate inflammation. For example, both PGE₂ and PGI₂ enhance endothelial barrier and protect against ventilator-induced lung edema.¹⁵ TXA₂ is reported to increase endothelial permeability and mediate intestinal reperfusion-induced pneumonia¹⁶ and allergic nasal inflammation.¹⁷ However, the mechanism by which PGD₂ modulates vascular permeability and inflammation remained unclear. We have previously shown that various types of endothelial cells express DP receptor and that PGD₂-DP signaling reduces vascular permeability in growing tumors.⁴ Here, by using croton oil-induced dermatitis as a model, we consistently demonstrate that DP agonism attenuates vascular permeability and that a host DP deficiency accelerates vascular hyperpermeability.

As described earlier, the DP receptor is categorized as a Gs protein-coupled receptor. Its activation results in intracellular cAMP accumulation, which plays a role in performing its biological functions. Here we examine the effect of DP stimulation on the downstream signaling pathway controlled

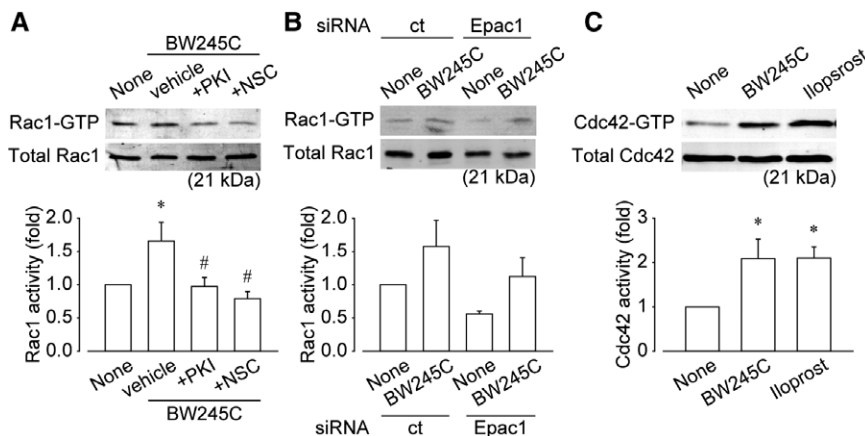


Figure 4. DP agonism activates Rac1 in a protein kinase A (PKA)-dependent, Epac1-independent manner. **A**, Effects of pretreatment with PKA inhibitory peptide (PKI) or NSC-23766 on BW245C-induced Rac1 activation (n=4). **P*<0.05 compared with none. #*P*<0.05 compared with BW245C-treated cells. Relative Rac1 activity was calculated as folds of none. **B**, Effect of Epac1 depletion on BW245C-induced Rac1 activation (n=4–6). Relative Rac1 activity was calculated as folds of none. **C**, Effect of BW245C and iloprost on Cdc42 activation (n=5–6). **P*<0.05 compared with none. Relative Cdc42 activity was calculated as folds of none. **Top** show typical picture and **bottom** show the summary. Data are presented as mean±SEM.

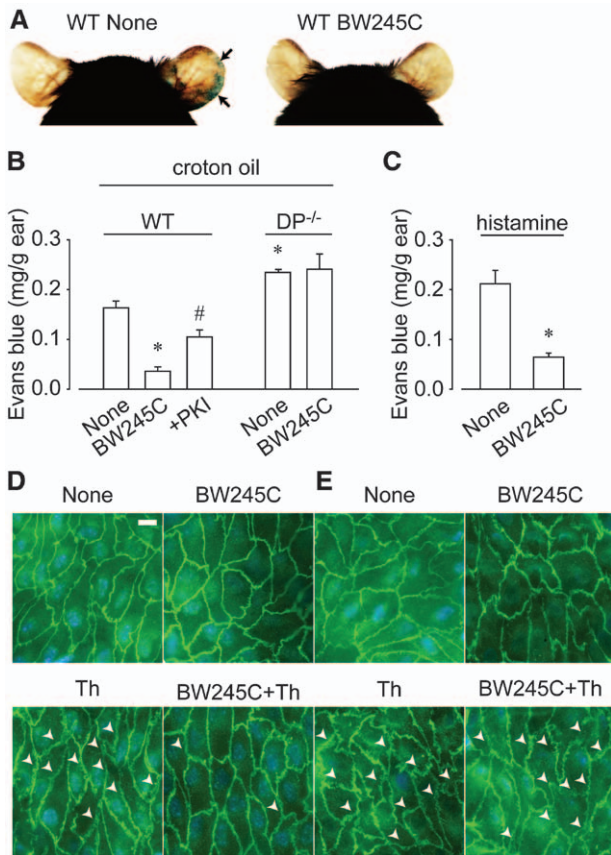


Figure 5. DP agonism inhibits croton oil- or histamine-induced ear hyperpermeability. **A**, Representative pictures of croton oil-induced Evans Blue extravasation; none (**left**, **arrows** indicate the extravasated Evans Blue) and BW245C-treatment (**right**). **B**, Effect of BW245C with or without protein kinase A inhibitory peptide (PKI) pretreatment on croton oil-induced Evans Blue extravasation in wild-type (WT) and $DP^{-/-}$ mice ($n=4-7$). $*P<0.05$ compared with WT none. $\#P<0.05$ compared with WT BW245C-treated mice. **C**, Effect of BW245C on histamine-induced Evans Blue extravasation in WT mice ($n=4-5$). $*P<0.05$ compared with none. **D** and **E**, Representative pictures of en face vascular endothelial (VE)-cadherin immunostaining in WT (**D**) and $DP^{-/-}$ (**E**) mice pulmonary artery endothelial cells. White **arrowheads** indicate VE-cadherin disassembly area. Scale bar: 10 μ m. Data are presented as mean \pm SEM.

by intracellular cAMP accumulation. PKA is an important effector of cAMP and is known to regulate endothelial permeability. We show here that DP receptor stimulation decreases endothelial permeability via PKA activation. There are some reports indicating that forskolin/rolipram-induced PKA activation promotes endothelial barrier function through Rac1 activation.^{18,19} Consistent with these reports, DP-mediated cAMP/PKA activation induces Rac1 activation. One interesting finding is that PKA inhibition abolishes BW245C-induced Rac1 activation (Figure 4A), whereas it exhibits only a partial attenuation of DP-mediated barrier enhancement (Figure 3C). These suggest the contribution of a PKA- or a Rac1-independent signaling pathway. Our data suggest the implication of Cdc42, another member of Rho family GTPases in DP-mediated barrier enhancement (Figure 4C). However, the detailed mechanism of Cdc42 activation remains unclear.

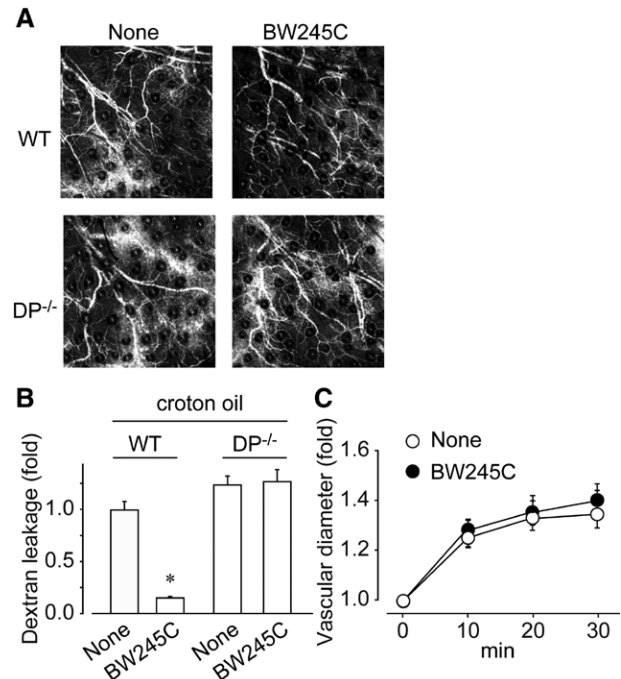


Figure 6. DP agonism inhibits croton oil-induced ear hyperpermeability but not vasodilation. **A**, Representative pictures of FITC-dextran extravasation induced by croton oil application in wild-type (WT) and $DP^{-/-}$ mice with or without BW245C-pretreatment. **B**, Effect of pretreatment with BW245C on croton oil-induced FITC-dextran leakage in WT and $DP^{-/-}$ mice ($n=5$). $*P<0.05$ compared with WT none. **C**, Effect of pretreatment with BW245C on croton oil-induced vasodilation in WT mice ($n=5$). Data are presented as mean \pm SEM.

Fukuhara et al⁹ first revealed that activation of another effector of cAMP, Epac, promotes endothelial barrier function. Subsequent studies showed that the barrier-enhancing effect of ANP, forskolin, and PGE₂ depends at least in part on Epac-dependent Rac1 activation.^{15,20,21} In contrast, our observations show that gene depletion of Epac1 reduces Rac1 activity and endothelial barrier integrity under nonstimulatory conditions but did not affect BW245C-induced activation in HUVECs. Previous reports showed that PKA can respond to nanomolar concentration of cAMP,²² whereas Epac1 requires micromolar levels of cAMP to be activated.²³ BW245C-induced cAMP elevation may be sufficient to activate PKA, but not to activate Epac1.

cAMP signaling is known to attenuate the intracellular concentration of Ca²⁺ ([Ca²⁺]_i)²⁴ which is required for endothelial cell contraction and barrier disruption. We examine how DP agonism influences endothelial [Ca²⁺]_i. Although treatment with BW245C (0.3 μ mol/L) slightly decreases [Ca²⁺]_i under nonstimulatory conditions, it does not affect 0.1 U/mL thrombin-induced [Ca²⁺]_i elevation (Figure III in the online-only Data Supplement). This observation may exclude the possibility that modulation of [Ca²⁺]_i contributes to DP-mediated barrier enhancement.

Rac1 acts as the convergent point of many endothelial barrier enhancing signaling pathways. We here show that Tiam1/Rac1 activation plays a critical role on DP-mediated barrier enhancement (Figure 2). Combined with the fact

that Tiam1 have a consensus PKA phosphorylation site,²⁵ we conclude that cAMP/PKA/Tiam1/Rac1 signaling pathway mediated the endothelial barrier enhancement induced by DP agonism. There are numerous reports indicating that Rac1 reorganizes the actin cytoskeleton and adherens junction.²⁶ These actin cytoskeleton rearrangements presumably increase cellular centrifugal tension and adherens junction stability. Consistent with previous reports, DP-mediated Rac1 activation induced cortical actin rim formation, VE-cadherin membrane translocation, and endothelial barrier enhancement. These strongly indicate adherens junction enhancement.

As well as endothelial barrier formation, blood flow is a critical factor determining vascular leakiness in vivo. Indeed, histamine causes vasodilation and subsequent hyperpermeability, whereas phenylephrine contracts vessels, restricts blood flow, and in turn relieves hyperpermeability.²⁷ However, DP agonism did not influence the oil-induced vascular dilation (Figure 6C). These allow us to conclude that endothelial barrier is a major target of DP stimulation in vivo as well.

Conclusion

We show that PGD₂-DP signaling decreases inflammation-associated vascular hyperpermeability through cAMP/PKA/Tiam1-dependent but Epac1-independent Rac1 activation. This study provides a well-supported indication that PGD₂-DP signaling can be a novel and productive therapeutic target for inflammatory diseases.

Sources of Funding

This work was supported by Grant-in-Aid for JSPS Fellows to Koji Kobayashi and Yoshiki Tsubosaka, Grant-in-Aid for Young Scientists (A) and Grant-in-Aid for challenging Exploratory Research from Japan Society for the Promotion of Science, Takeda Science Foundation, The Naito Foundation, SENSHIN Medical Research Foundation, and The Pharmacological Research Foundation to Takahisa Murata.

Disclosures

None.

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