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Prostaglandin D_2 strengthens human endothelial barrier by activation of E-type receptor 4

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Abstract: Life-threatening inflammatory conditions such as acute respiratory distress syndrome or sepsis often go hand in hand with severe vascular leakage. During inflammation, endothelial cell integrity and intact barrier function are crucial to limit leukocyte and plasma extravasation. Prostaglandin D_2 (PGD_2) is a potent inflammatory lipid mediator with vasoactive properties. It has been suggested that PGD_2 is involved in the regulation of endothelial barrier function; however, it is unclear whether this is also true for primary human pulmonary microvascular endothelial cells. Furthermore, as PGD_2 is a highly promiscuous ligand, we set out to determine which receptors are important in human pulmonary endothelial cells. In the current study, we found that PGD_2 and the DP1 agonist BW245c potently strengthened pulmonary and dermal microvascular endothelial cell barrier function and protected against thrombin-induced barrier disruption. Yet surprisingly, these effects were mediated only to a negligible extent via DP1 receptor activation. In contrast, we observed that the EP4 receptor was most important and mediated the barrier enhancement by PGD_2 and BW245c. These data demonstrate a novel mechanism by which PGD_2 may modulate inflammation and emphasizes the role of EP4 receptors in human endothelial cell function.

Keywords: Prostaglandin D_2, BW245c, prostanoid signalling, EP4 receptor, endothelial barrier, barrier disruption, microvascular endothelium

1. Introduction

Throughout the body, endothelial cells form a solid barrier between circulation and interstitial space, thereby, keeping extravasation of plasma proteins or circulatory cells at bay. This strong barrier is enforced by inter-endothelial tight and adherens junctions, i.e. vascular endothelial (VE-) cadherin, as well as focal adhesion points, which tether endothelial cells to extracellular matrix components [1]. Regulation of the endothelial barrier is maintained by a range of exogenous and endogenous agents [2] and is particularly critical in limiting inflammatory reaction in tissues constantly exposed to external factors and pathogens such as lung and skin [3]. Dysfunction of endothelial cell lining results in aggravated oedema, leukocyte extravasation and inflammatory responses. In extreme cases, such as anaphylactic responses, systemic breakdown of vascular integrity may occur [2,4]. Notably, plasma and leukocyte extravasation primarily occurs in the smallest vessels [5], making microvascular endothelial cells an interesting target for barrier-enhancing therapies.

Prostaglandins (PGs) are inflammatory lipid mediators released during acute and chronic inflammation by various leukocytes and structural cells [6]. The closely related mediators prostaglandin E_2 and D_2 are highly abundant during inflammation, however they often have opposing roles. Both, pro- and anti-inflammatory effects of PGD_2 and its G-protein-coupled receptors

(GPCRs) DP1 and DP2 have been reported. Notably, PGD_2-mediated effects vary strongly among animal species, tissues and experimental settings [7,8].

In addition to recruitment and activation of immune cells, prostaglandins influence the vascular response during inflammation. In a mouse model of systemic anaphylaxis, DP1 agonism reduces vascular hyper-permeability [9]. Furthermore, this study also showed that genetic deletion of the DP1 receptor increases vascular permeability. Accordingly, Murata et al. demonstrated that PGD_2, DP1 agonism and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) attenuate leukocyte infiltration by enhancing endothelial barrier in a murine model of acute lung injury [10]. Consistently, PGD_2 as well as DP1 have been shown to promote endothelial barrier function in human dermal microvascular endothelial cells via cyclic AMP-PKA-Tiam1-Rac1 [11] and reduce vascular leakage in a mouse model of acute lung injury [12]. In addition to activating DP receptors in nano-molar concentrations, PGD_2 and its metabolite 15d-PGJ_2 also activate peroxisome proliferator-activated receptor γ (PPARγ) in the micro-molar range [13,14]. Loss of PPARγ signalling in endothelial cells causes vascular leakage in mice [15] and reduced expression of tight junction proteins in human brain microvascular endothelial cells [16]. Furthermore, several studies suggest that PGD_2 in micro-molar range acts as a thromboxane receptor (TP) agonist. PGD_2-TP activation results in pulmonary vasoconstriction in sheep [7] and constriction of airways, pulmonary arteries and veins in guinea pigs [17]. Interestingly, the DP1 agonist BW245c caused systemic vasorelaxation in rats [18] and DP1 receptor blockade could reduce niacin-induced facial flushing in patients [19]. Further, DP1 and the PGE_2 receptor EP2 are closely related as they developed by gene tandem duplication [20]; therefore, PGD_2 can to some extent activate EP2 receptors [21]. These studies highlight the promiscuity of PGD_2 in prostaglandin receptor activation pertinent to the regulation of vascular function.

PGE_2 is structurally highly similar to PGD_2 but exerts different functions through activation of four GPCRs, E-type receptor 1-4 (EP 1-4). While endothelial barrier function is not subject to regulation by EP1 and EP3 receptor activation [22], we have shown that PGE_2 enforced human pulmonary microvascular endothelial barrier function via EP4 activation and reduced leukocyte adhesion to endothelial cells [23]. Additionally, PGE_2-EP4 stimulation protected against thrombin-induced barrier disruption in human endothelium [23] and ameliorated acute lung injury in mice [24].

PGD_2 has the capacity to bind to nearly all prostanoid receptors albeit with low affinity [25], which may cause additional synergistic or opposing effects to DP receptor activation. It is still unclear, why PGD_2 acts pro-inflammatory in one setting while it has anti-inflammatory actions in others. A thorough understanding of which receptors are targeted by PGD_2 on different cell types and which dose range triggers certain effects, will help to augment therapeutic development. Some studies have already indicated that PGD_2 may bind with low affinity to EP4 receptors in human embryonic kidney (HEK) cells overexpressing EP4 receptor [25,26] which was supported by *ex vivo* experiments investigating rabbit saphenous vein relaxation [27]. To date, it has not been examined, whether PGD_2/EP4 interaction has any physiological relevance in primary human cells.

Here we report, that PGD_2 increases barrier function and protects against thrombin-induced barrier disruption in primary human pulmonary and dermal microvascular endothelial cells as well as in pulmonary artery endothelial cells by activating EP4 and only to a small extent by DP1 receptors.

2. Materials and Methods

Endothelial cell culture. Human pulmonary microvascular endothelial cells (HPMEC, Lonza, Basel, Switzerland or PromoCell, Heidelberg, Germany), dermal microvascular endothelial cells (HDMEC, PromoCell, Heidelberg, Germany) or pulmonary artery endothelial cells (HPAEC, Lonza, Basel, Switzerland) were cultured in corresponding medium (EGM MV2 with microvascular endothelial cell supplementary kit C-22121, PromoCell or CC-4147, Lonza) in T75 Corning CellBind flasks pre-coated with attachment factor solution (PeloBiotech, Bavaria, Germany). A list of endothelial cell donors used for experiments can be found in Table S1. Cells were passaged when 90 % confluence was reached, detached with Trypsin/EDTA solution from respective company (PromoCell or Lonza) while cells from passage 4 to 9 were used for experiments.

Electric Impedance Cell-substrate Sensing (ECIS). Endothelial cells were seeded at a density of 60 000 – 80 000 / 400 µl of complete medium / well onto 8W10E+ polycarbonate arrays (AppliedBiophysics, NY, USA) pre-coated with 10 mM L-cysteine in sterile water, followed by coating with 1 % gelatin solution and grown until confluence for 2 to 3 days. An ECIS® Z-Theta device (AppliedBiophysics) was used for online monitoring of resistance changes within the cellular monolayer. Prior to treatment, cells were serum starved in EBM-2 basal medium (PromoCell or Lonza) supplemented with 2 % fetal calf serum (FCS; ThermoFisher Scientific, Massachusetts, USA) for 1 h, followed by baseline measurement for 2 h. Endothelial barrier function was determined after pre-treatment with antagonists for 30 min followed by PGD_2 or other agonists dissolved in EBM-2 basal medium with 2 % FCS. A list of all agonists and antagonists used in this study are summarized in Table S2. Each treatment was performed in duplicate and normalized resistance for each well was recorded every 30 s for 20 h (4.5 h displayed for clarity) after agonist addition. To evaluate the protective capacity of PGD_2 and BW245c, cells were seeded into polycarbonate arrays with gold electrodes as described above. After 1 h starvation and 2 h baseline measurements, cells were pre-treated with vehicle or 300 nM ONO-AE3-208 for 30 minutes followed by stimulation with indicated concentration of vehicle, PGD_2, BW245c or PGE_2 for 15 minutes before barrier disruption was initiated by addition of 0.5 U/ml of recombinant human thrombin (Sigma-Aldrich, Missouri, USA, T7009-100).

Thrombin barrier disruption assay. Lab-Tek II CC^2 8-chamber well slides (ThermoFisher Scientific) were pre-coated with 1 % gelatine solution followed by seeding of 60 000 – 80 000 endothelial cells / well. When confluence was reached, cells were serum starved in EBM-2 basal medium with 2 % FCS for 30 minutes followed by incubation with EP4 receptor antagonist ONO-AE3-208 or vehicle for 20 minutes. Subsequently, endothelial cells were treated with indicated concentrations of PGD_2, PGE_2 or selective agonists for 15 min before cells were challenged with 0.5 U/ml recombinant human thrombin (15 min). All incubations were performed at 37 °C, 5 % CO_2 and humidified atmosphere. Cells were washed once with pre-warmed Hepes-buffered saline and fixed in 3.8 % formalin solution (CarlRoth, Karlsruhe, Germany) for 10 minutes at room temperature.

Immunofluorescence staining. All steps were performed at room temperature. For evaluation of EP4 or DP1 receptor expression, cells were serum starved in EBM MV2 basal medium with 2 % FCS for 1 h before fixation in 3.8 % formalin solution (10 min). Cell monolayer was gently washed three times with PBS and non-specific binding was blocked by incubation with 10 % normal goat serum (Sigma Aldrich) and 1 % bovine serum albumin in phosphate buffered saline for 30 minutes. Subsequently, cells were incubated with primary rabbit anti-DP1 antibody (Origene, Maryland, USA, TA340654, 1 to 200 in PBS with 1 % goat serum) for 1 h. After washing with PBS, cells were permeabilized with 0.1% Triton-X-100 in PBS for 10 minutes, followed by incubation with blocking solution for 30 minutes. Next, cells were stained with primary mouse anti-EP4 (1 to 200, Santa Cruz, TX, USA, sc55596, C-4) for 1 h. For evaluation of the thrombin barrier disruption assay, cells were permeabilized with 0.1 % TritonX-100 in PBS after fixation. Non-specific binding was blocked for 30 minutes before cells were incubated with primary mouse anti-human VE-cadherin antibody (1 to 200, sc-9989, Santa Cruz, TX, USA) for 1 h. In the last step, cells were incubated with secondary AF488-conjugated goat anti-mouse antibody (VE-cadherin), AF488-conjugated goat anti-rabbit (DP1) or AF647-conjugated goat anti-mouse (EP4) (1 to 500, ThermoFisher Scientific) and Texas Red-X-conjugated phalloidin (1 to 40, ThermoFisher Scientific) for 30 minutes. Slides were mounted with VectaShield / DAPI fluorescence mounting medium and images were taken using an Olympus IX70 fluorescence microscope with an Olympus UPlanApo-20x or 60x (oil immersion) lens.

Quantitative evaluation of endothelial monolayer integrity. To visualize the protective effect of PGD_2, BW245c, DK-PGD_2 and PGE_2, 5 images per well were taken (20x magnification) of 5 independent experiments by one operator, while scoring of barrier integrity was performed by another operator unfamiliar with experimental treatments. Fiji ImageJ software was used for counting and to reduce the background noise. Cropped cells at image edges were excluded from quantification. The percentage of cells with disrupted barrier was evaluated using VE-cadherin

staining at cell periphery. In vehicle-treated cells, peripheral VE-cadherin forms a thin, but continuous line between neighbouring cells. Stimulation with a barrier-enhancing agent such as PGE_2 strengthens this VE-cadherin junctional zone, which appears now brighter, wider and more uniform. At the same time, actin fibres are assembled at cell periphery to support VE-cadherin junctional complexes. Challenge with a barrier-disrupting agent such as thrombin leads to Rho-dependent stress fibre formation and Rac-dependent cell rounding and retraction. In the immunofluorescence images, this was observed as reduction of cortical F-actin staining, appearance of F-actin stress fibres throughout the cell body and, in its most severe form, stress fibres close to the nuclei. Further, actin polymerization causes cellular contraction, which causes intercellular gaps. Therefore, three parameters were considered to evaluate endothelial monolayer integrity: 1) percentage of cells with stress fibres covering >80 % of cytoplasmic area and/or stress fibres close to the nucleus, 2) number of inter-endothelial gaps normalized to total number of nuclei per field and 3) VE-cadherin staining at cell periphery, where a cell with less than 60 % continuous circumferential VE-Cadherin staining was considered as 'cell with disrupted barrier'. One data point corresponds to the mean value of 5 images per well (>250 cells). Schematic drawing of F-actin and VE-cadherin changes and one example to demonstrate how cells were counted can be found in Figure 2A and B, respectively.

EP4-knock down in primary human microvascular endothelial cells. EP4 knock-down in human pulmonary microvascular endothelial cells was conducted as described previously [22]. Briefly, HPMEC were seeded onto gelatine-coated 6 well plates (Corning CellBind) and transfected for 48 h with Lipofectamine RNAiMAX (ThermoFisher Scientific) according to the manufacturer's instructions when they reached 60-70 % confluence. Each experimental set-up consisted of untreated, mock-transfected (Lipofectamine only), non-targeting control siRNA (50 nM) and specific PTGER4 siRNA (50 nM PTGER4 FlexiTube-GeneSolution, Quiagen, Hilden, Germany) transfected cells as well as additional wells (control and EP4-specific siRNA) for knock-down control via qPCR. After 48 h transfection, cells were either collected in TriReagent for mRNA extraction or detached using trypsin/EDTA and seeded onto gelatine-coated 8W10E+ polycarbonate ECIS arrays. Cells were left to adhere in EBM-MV2 basal medium with 2 % FCS for 5 h and ECIS experiments were conducted as described above. For evaluation of barrier integrity after EP4 receptor knock down, cells were seeded directly into gelatine-coated Lab-Tek II CC^2 8-chamber well slides (60 000 cells per well) in antibiotic-free complete medium overnight and transfected on the following day (500 µl per well; 50 nM siRNA) for 48 h. Subsequently, cells were starved for 1 h in EBM-MV2 basal medium with 2 % FCS before stimulation with vehicle, 3 µM PGD_2, 3 µM BW245c or 100 nM S1P for 15 minutes. Cells were fixed in 3.7 % formalin solution in PBS for 10 minutes at room temperature and VE-cadherin and F-actin stained as described above.

Real time quantitative PCR. For evaluation of knock-down efficiency, cells were collected after 48 h transfection in TriReagent (Sigma Aldrich) and mRNA extracted followed by purification with an RNAeasy Kit (Qiagen, Hilden, Germany). 1 µg of RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad, CA, USA) according to the manufacturer's instructions. SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) in combination with PrimePCR™ SYBR® Green Assay primers for PTGER4 and GAPDH (Bio-Rad) were used for RT-PCR. Samples were run in duplicates, PTGER4 normalized to GAPDH Cq values and results are shown as percentage of control siRNA. To evaluate DP1, DP2 and EP4 receptor mRNA expression levels in HPMEC, HDMEC and HPAEC, RNA was extracted and cDNA generated from unstimulated cells as described above. Validated PrimePCR™ SYBR® Green Assay primers for human PTGER4, PTGDR, PTGDR2 and GAPDH (Bio-Rad) were used. Additionally, we isolated RNA and generated cDNA from human peripheral blood monocytes to ensure functionality of DP1 and DP2 primer pairs. Peripheral blood monocytes were obtained from healthy human donors, which was approved by the Institutional Review Board of the Medical University of Graz (EK 17-291 ex 05/06). All volunteers signed an informed consent. Monocytes were enriched using the Monocyte isolation Kit II (Miltenyi Biotech, Bergisch Gladach, Germany) and RNA extracted and cDNA generated as described above.

Western blotting. Cell lysates were collected in 100 µl protein lysis buffer (10 mM Hepes, 1 mM EDTA, 1 % Triton-X, 1 mM sodium-orthovanadate, 7.5 µl protease inhibitor cocktail (Sigma)) and sonicated (4 cycles at 40% power for 10s each) followed by centrifugation at 12 000 rpm for 10 min at 4°C and the supernatant was used for Western blotting. Total protein was determined using a BCA assay (ThermoFisher Scientific) according to the user manual and approximately 20 µg of protein was loaded onto a precast gel (Novex WedgeWell Tris-Glycine gels, 4-20 %, XP04205, ThermoFisher Scientific). The gel was run at 225 V for 45 min, and then washed for 10 min in distilled water, protein bands were transferred onto a PVDF membrane with help of an iBlot gel transfer device (Thermofisher Scientific). Subsequently, unspecific binding was blocked with 5 % milk in TBST buffer on a shaker at room temperature for 1 h. Subsequently, the membrane was incubated with primary rabbit anti-human EP4 (Santa Cruz, sc-20677, 1 to 200) in 5 % milk at 4 °C overnight. Next day, the membrane was incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit HRP-conjugated antibody (Jackson ImmunoResearch, 111-035-045, 1/5000) or for 1.5 h. After washing, bands were visualized by incubation for 5 min with Clarity™ Western ECL Blotting Substrate (BioRad) and subsequently evaluated with a BioRad chemiluminescence detector and corresponding Software (ImageViewer). After detection, the membrane was washed for 30 min with stripping buffer (65.2 mM Tris/HCl with 2 % SDS pH 6.9 with 100 nM β-mercaptoethanol) at 50 °C with shaking, washed three times with TBST buffer for 10 min, blocked for 30 min at RT with 5 % milk and subsequently incubated with primary rabbit anti-human DP1 (CaymanChem, 101640, 1/200), primary rat anti-DP2 (SantaCruz, BM16, 1/200) or mouse anti-human beta-actin (BioTechne, Minnesota, USA, NB600-501, 1/5000) and corresponding secondary antibody on the following day (horseradish peroxidase-conjugated secondary goat anti-mouse (JacksonImmunoResearch, 115-035-062), goat anti-rabbit (Jackson ImmunoResearch, 111-035-045, 1/5000) or goat anti-rat (JacksonImmunoResearch, 112-035-003) for 1.5 h.

Platelet aggregation. Isolation of platelets from human donors was approved by the Institutional Review Board of the Medical University of Graz (EK 17-291 ex 05/06). All volunteers signed an informed consent. Washed platelets were obtained from platelet rich plasma (citrated blood) from healthy donors by centrifugation at 1000 x g for 15 minutes without break, followed by two washing steps in wash buffer (140 mM NaCl, 10 mM $NaHCO_3$, 2.5 mM KCl, 0.9 mM $Na_2HPO_4 * 2 H_2O$, 2.1 mM $MgCl_2$, 22 mM $C_6H_5Na_3O_7$, 0.055 mM D(+)-Glucose monohydrate and 0.35 % bovine serum albumin; pH 6.5) as previously described [28]. Platelets were resuspended in Tyrode buffer and platelet aggregation was determined using the 4-channel platelet aggregometer APACT4004 (LabiTec, Ahrensburg, Germany). Aggregation of washed platelets was recorded after addition of pro-aggregatory stimulation with adenosine diphosphate (ADP; Probe & Go, Osburg, Germany, 5-20 µM to induce ~30-40% aggregation) with constant stirring at 37 °C. Platelets were pre-treated with vehicle (EtOH) or 100 nM ONO-AE3-208 for 10 minutes followed by stimulation with 30 nM PGD_2 or vehicle (EtOH) for 5 minutes before stimulation with ADP in the presence of 1 µg/ml of fibrinogen (F4129-1G, Fraction I, type III: from human plasma, Sigma Aldrich, Missouri, USA). Aggregation was measured as increase in light transmission for 300 seconds and data were expressed as percent of maximum light transmission (maximum percentage of total aggregation), with non-stimulated platelet-rich plasma being 0 % and platelet-poor plasma 100 %.

Statistical analysis. Data are shown as mean and SEM for n observations, where n denotes independent experiments. Comparisons of groups were performed as appropriate with GraphPad Prism 8 software using 1-way ANOVA followed by Tukey's post hoc test or 2-way ANOVA for repeated measurements followed by Fisher's LSD or Tukey's post hoc test to determine the levels of significance for each group. Probability values of $P < 0.05$ were considered as statistically significant.

3. Results

PGD_2 and the DP1 agonist BW245c but not the DP2 agonist DK-PGD_2, enhance human microvascular endothelial barrier in a concentration-dependent manner

At a concentration range of 0.3 to 3 µM, both PGD_2 and the DP1 agonist BW245c increased the electrical resistance of primary human dermal microvascular endothelial cells in a concentration-dependent manner, while the DP2 agonist DK-PGD_2 had no effect (Figure 1A, B, C). In line with this, VE-cadherin staining increased upon PGD_2 and BW245c treatment (Figure 1D. To determine whether this was also true for other types of endothelial cells, we performed the same experiments with primary human pulmonary microvascular endothelial cells (HPMEC). In HPMEC, PGD_2 and BW245c-treatment produced similar but somewhat smaller effects in magnitude (Figure 1E,F,G) and increased the expression of VE-cadherin (Figure 1H).

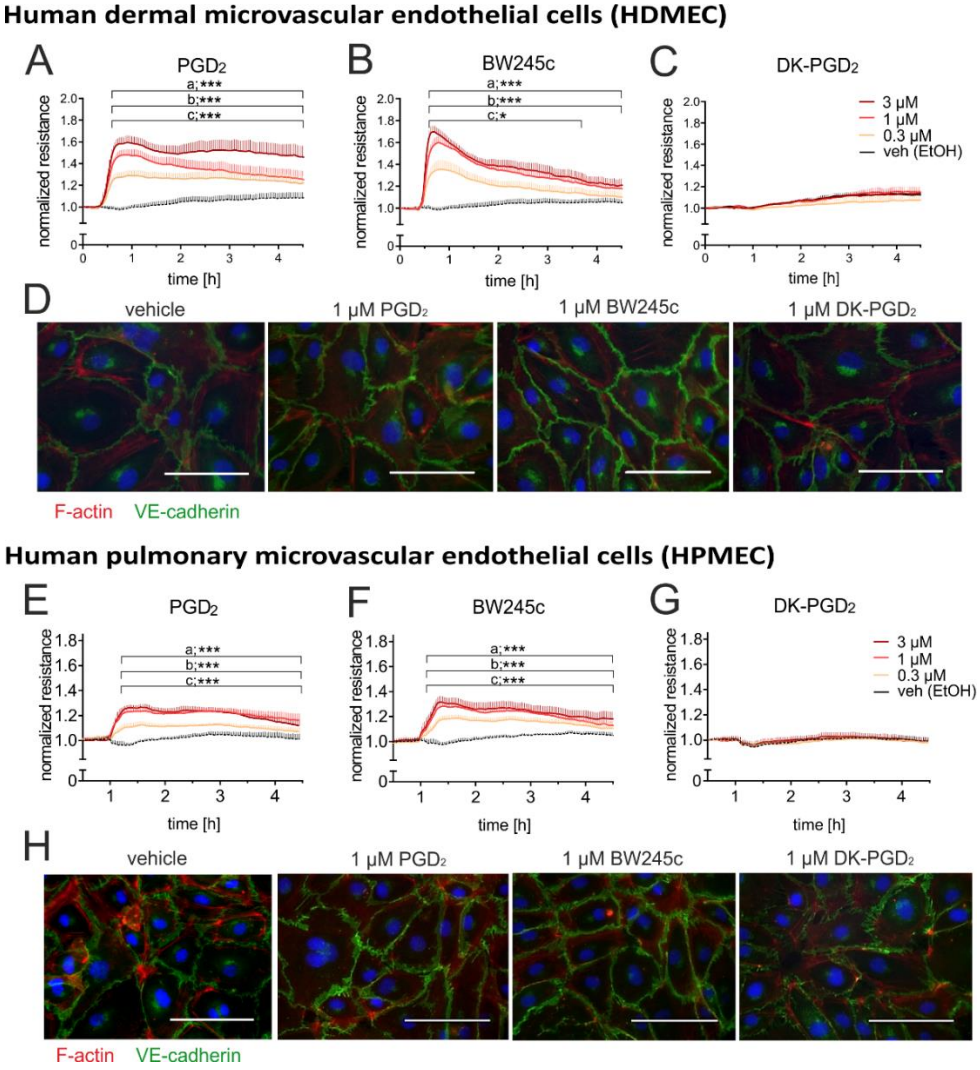

Figure 1 PGD_2 and DP1 agonist BW245c but not DP2 agonist DK-PGD_2 increase human microvascular endothelial barrier in a concentration-dependent manner. Human dermal (HDMEC) or pulmonary (HPMEC) microvascular endothelial cells were seeded onto 8W10E+ polycarbonate ECIS arrays and serum starved for 3 h. Changes in endothelial monolayer resistance, which is proportional to endothelial barrier function, were recorded in real time with an ECIS® Z-Theta device and the mean of duplicate measurements was plotted. In HPMECs (A) PGD_2 as well as (B) DP1 agonist BW245c increased the resistance of endothelial monolayers. (C) Stimulation with DP2 agonist DK-PGD_2 did not change the resistance. (E) PGD_2 and (F) BW245c, but not (G) DK-PGD_2, also increased the resistance in HPMEC. (D) and (H) Immunofluorescence staining of VE-cadherin and F-actin in HDMEC and HPMEC, respectively, revealed a stronger VE-cadherin barrier in PGD_2 and BW245c-treated cells. Images are representative for 5 independent experiments (scale bar 100 µm). Data are shown as mean + SEM, n = 5. Two-way ANOVA for repeated measurements with Tukey's

post hoc test, * p<0.05 and *** p<0.001, (a) veh (EtOH) vs. 3 µM agonist, (b) veh (EtOH) vs. 1 µM agonist, (c) veh (EtOH) vs. 0.3 µM agonist.

PGD$_2$ and DP1 agonist BW245c protect human pulmonary microvascular endothelial cells against thrombin-induced barrier disruption

During inflammation, exposure to barrier-disruptive agents such as thrombin, bacterial lipopolysaccharide or histamine facilitates extravasation of leukocytes and oedema. In intact microvessels, F-actin filaments align at the cellular periphery and VE-cadherin forms a tight barrier between neighbouring endothelial cells, as can be seen in the scheme in Figure 2A (left). Thrombin as a strong inducer of endothelial permeability caused the formation of F-actin stress fibres, cellular contraction and dissociation of the VE-cadherin barrier (Figure 2A, right). As both PGD$_2$ and BW245c increased microvascular endothelial barrier function, we investigated whether pre-treatment of microvascular endothelial cells with PGD$_2$ and BW245c could protect against thrombin-induced barrier disruption. Indeed, pre-treatment of HPMEC with 1 µM PGD$_2$ or 1 µM BW245c significantly reduced the percentage of cells with a disrupted VE-cadherin barrier (Figure 2E). DK-PGD$_2$ conferred no protective effect. Furthermore, pre-treatment with PGD$_2$ and BW245c slightly reduced actin stress fibre formation (Figure 2F) and the number of inter-endothelial gaps (Figure 2G). For all experiments, treatment with 300 nM PGE$_2$ was performed in parallel as positive control. Similar results were obtained with HDMEC (Figure S1).

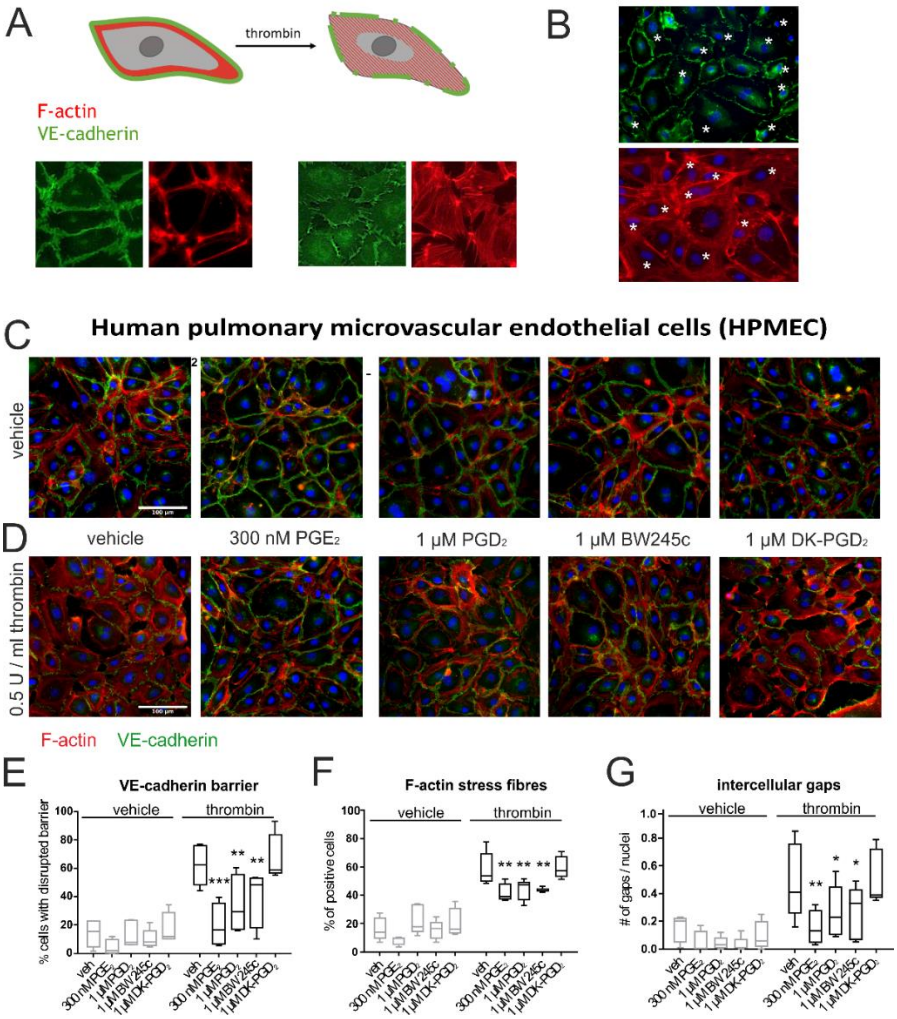

Figure 2. PGD$_2$ and DP$_1$ agonist BW245c but not DP2 agonist DK-PGD$_2$ protect HPMECs against thrombin-induced barrier disruption. (A) Schematic drawing of peripheral F-actin fibres and VE-cadherin at steady state (left) and after treatment with a barrier disrupting agent (right). (B)

Evaluation strategy of cells judged as positive for stress fibres or disrupted barrier indicated with an asterisk. (C, D) Representative images for 5 independent experiments of VE-cadherin and F-actin stained, confluent HPMECs stimulated with vehicle (EtOH), 1 µM PGD_2, BW245c, DK-PGD_2 or 300 nM PGE_2 for 15 minutes followed by incubation with (C) vehicle (a.d.) or (D) 0.5 U/ml thrombin for 15 minutes (scale bar 100 µm). Extent of barrier disruption was evaluated by quantifying (E) the percentage of cells with disrupted barrier, (F) the percentage of cells with actin stress fibres and (G) the ratio of intercellular gaps per nuclei. Data are displayed as box and whisker plot, n = 5. Two-way ANOVA with Fisher's LSD post hoc test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ difference between veh / thrombin vs. agonist.

DP1 antagonism but not DP2 antagonism partially reduces PGD_2-induced barrier enhancement

Since the DP1 agonist mimicked all PGD_2 barrier effects, we tested whether DP1 receptor activation is in fact responsible for the barrier protective effect using a DP1 antagonist. Interestingly, blockade of DP1 receptor with BWA868c, only partly reduced the PGD_2-induced increase in HPMEC resistance (Figure 3A), while second DP1 antagonist, MK0524, did not show any inhibitory effect (Figure S2). As expected, DP2 blockade with Cay10471 did not affect PGD_2-induced barrier enhancement (Figure 3B). DP1 or DP2 antagonism had similar effects in HDMEC (Figure S3). Under certain conditions, DP1-DP2 interaction has been shown to influence downstream signalling [29]; however, synergistic blockade of DP1 and DP2 receptors on HPMEC also did not reverse the barrier increase (Figure 3C).

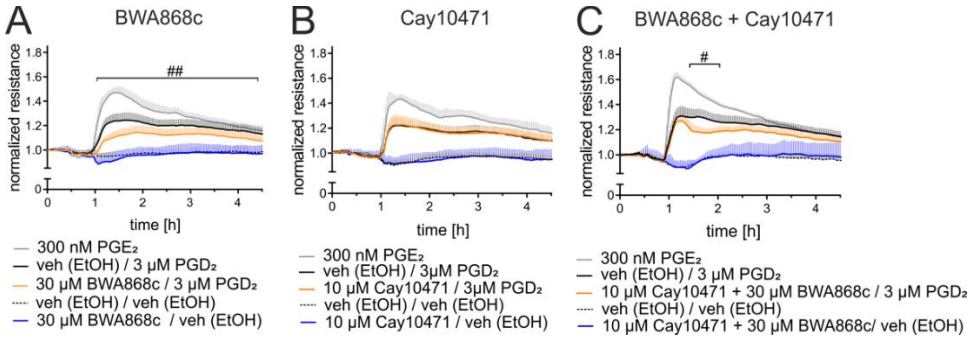

Figure 3. Blockade of DP1 and DP2 receptors does not reverse the PGD_2-induced barrier increase. (A) Pre-treatment of HPMEC with 30 µM of the DP1 antagonist BWA868c for 30 minutes slightly, but significantly diminished the PGD_2-induced barrier increase. (B) Pre-treatment with 10 µM of the DP2 antagonist Cay10471 did not affect PGD_2-induced increase in resistance. (C) A combination of BWA868c and Cay10471 had only a minor effect. Data are displayed as mean + SEM, n = 5, two-way ANOVA for repeated measurements with Tukey's post hoc test, # $p<0.05$, ## $p<0.01$ difference between veh (DMSO) / 3 µM PGD_2 vs. antagonist / 3 µM PGD_2.

PGD_2 does not enhance HPMEC barrier function through activation of PPARγ, TP receptors or cyclooxygenases

It is known that PGD_2 and some of its metabolites are able to bind to PPARγ receptors and PPARγ is important for sustained endothelial barrier function [14,15,25]. Pre-treatment with the PPARγ antagonist T0070907 (30 µM) had a considerable, but transient, effect on endothelial barrier function, and diminished the increase of endothelial electrical resistance following PGD_2 stimulation (Figure 4A). In contrast, pre-treatment with 10 µM SQ 29,548, a TP receptor antagonist, did not prevent the PGD_2-induced increase in microvascular endothelial cell resistance (Figure 4B). Next, we excluded the possibility that the effects of PGD_2 may depend on, or be modified by, endogenously released prostaglandins. Accordingly, inhibition of cyclooxygenase-1/2 with 1 µM diclofenac did not affect the barrier response to PGD_2 (Figure 4C).

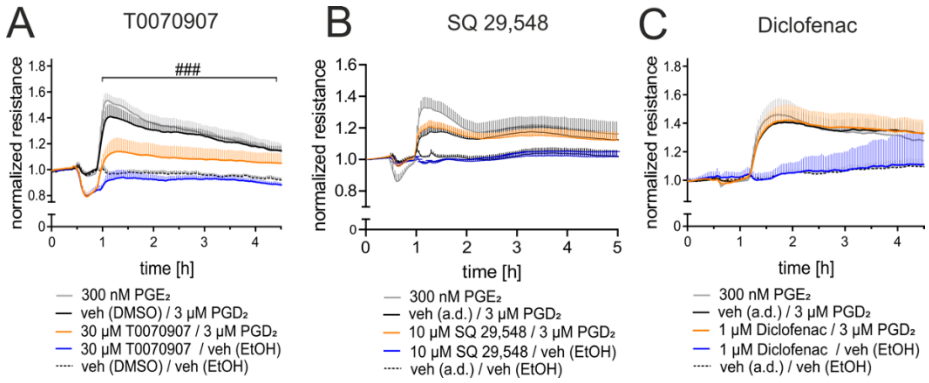

Figure 4. PGD_2 partially enhances microvascular barrier function through activation of PPARγ, but not via TP receptors and cyclooxygenases. (A) Pre-treatment with PPARγ antagonist T0070907 caused a transient drop of endothelial electrical resistance, and attenuated the PGD_2-induced barrier enhancement, while blockade of (B) TP receptors with 10 µM SQ 29,548 or (C) cyclooxygenase-1/2 with 1 µM diclofenac had no effect. Data are shown as mean + SEM, A and C: n = 5, B: n = 3, two-way ANOVA for repeated measurements with Tukey's post hoc test # $p<0.05$ difference between veh (DMSO) / 3 µM PGD_2 vs. antagonist / 3 µM PGD_2.

Human pulmonary microvascular endothelial cells express very low levels of DP1, but high levels of the EP4 receptor

Next, we evaluated DP1, DP2 and EP4 receptor mRNA expression levels in endothelial cells. Interestingly, human pulmonary microvascular (Figure 5A), dermal microvascular (Figure S4A) and pulmonary arterial endothelial cells (Figure S4B) cultured in respective complete medium expressed very low levels of DP1 and DP2 mRNA . In contrast, all cell types expressed high levels of EP4 receptor mRNA, as indicated by thresholds (Cq) reached several cycles earlier. In comparison, human peripheral blood monocytes showed higher mRNA levels of DP1 and, especially DP2, than endothelial cells (Figure S4C), but comparable levels of EP4 mRNA. In unstimulated HPMEC, Western blot suggested higher expression levels of EP4 than DP1 receptor protein (Figure 5B). More information about HPMEC donors shown in Figure 5B is provided in supplementary table 1. Finally, EP4 and DP1 receptor expression was visualized by immunofluorescence staining (Figure 5C). Additional representative images (60x magnification) for EP4 and DP1 receptor staining in HPMEC are shown in Figure S4D along with isotype and 2nd only controls (Figure S4E).

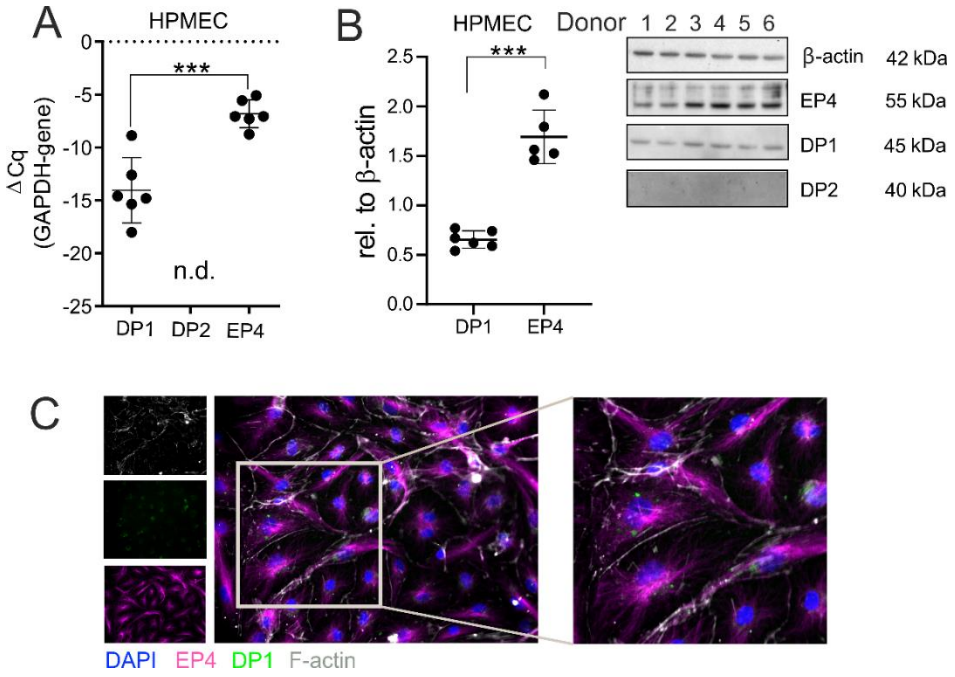

Figure 5. Human pulmonary microvascular endothelial cells express very low levels of DP1 and DP2 but higher levels of EP4 receptor mRNA and protein. (A) Receptor mRNA expression was quantified by real time PCR and (B) Western blot of unstimulated HPMEC. Data are shown as mean ± SD, n = 6, one-way ANOVA with Tukey's post hoc test (qPCR) or Student's t-test (WB), n.d. – non detectable, ***p<0.001. (C) Immunofluorescence staining (20x magnification) of EP4 and DP1 receptors in HPMEC (representative for 3 independent experiments).

Blockade of EP4 receptors abrogates PGD_2 and BW245c barrier enhancement and reverses protection against thrombin-induced barrier disruption.

Previously we described that PGE_2-EP4 activation has a strong barrier protective effect in HPMEC [23]. Notably, PGE_2 is about 10x more potent than PGD_2 and BW245c in strengthening endothelial cell resistance but spatial-temporal responses are comparable, i.e. a high peak shortly after addition of stimulating agent followed by a slightly lower but sustained increase in endothelial resistance for several hours. Considering that EP4 receptors are responsible for PGE_2-induced barrier enhancement and due to their high expression levels in human endothelial cells, we decided to analyse the involvement of this receptor in the effects we have seen for PGD_2 and BW245c. Indeed, blockade of EP4 receptors with 300 nM ONO-AE3-208, a specific EP4 receptor antagonist, completely abolished PGD_2 as well as BW245c-induced barrier stimulation in HPMEC (Figure 6A and 6B) and in HDMEC (Figure S5A and S5B). To corroborate this result further, we used another EP4 antagonist, GW627368X, which also strongly inhibited the PGD_2- as well as BW245c-induced barrier enhancement (Figure S6). In vehicle pre-treated cells, there was an increase in VE-cadherin staining at the cell periphery after PGD_2 (Figure 6C) or BW245c (Figure 6D) treatment, however, had the cells been pre-treated with 300 nM ONO-AE3-208, this effect was not seen any longer. Inhibition of EP4 receptor signalling also ablated the protective effect of PGD_2 against thrombin disruption of the barrier as shown with electrical resistance measurement (Figure 6E and F) and immunofluorescent staining of VE-cadherin and F-actin (Figure 6G and H). In contrast, ONO-AE3-208 did not attenuate sphingosine-1-phosphate induced barrier enhancement (Figure S7). The selectivity of ONO-AE3-208 for EP4 receptors was also underpinned by its lack of effect on the PGD_2-induced inhibition of platelet aggregation (Figure S8).

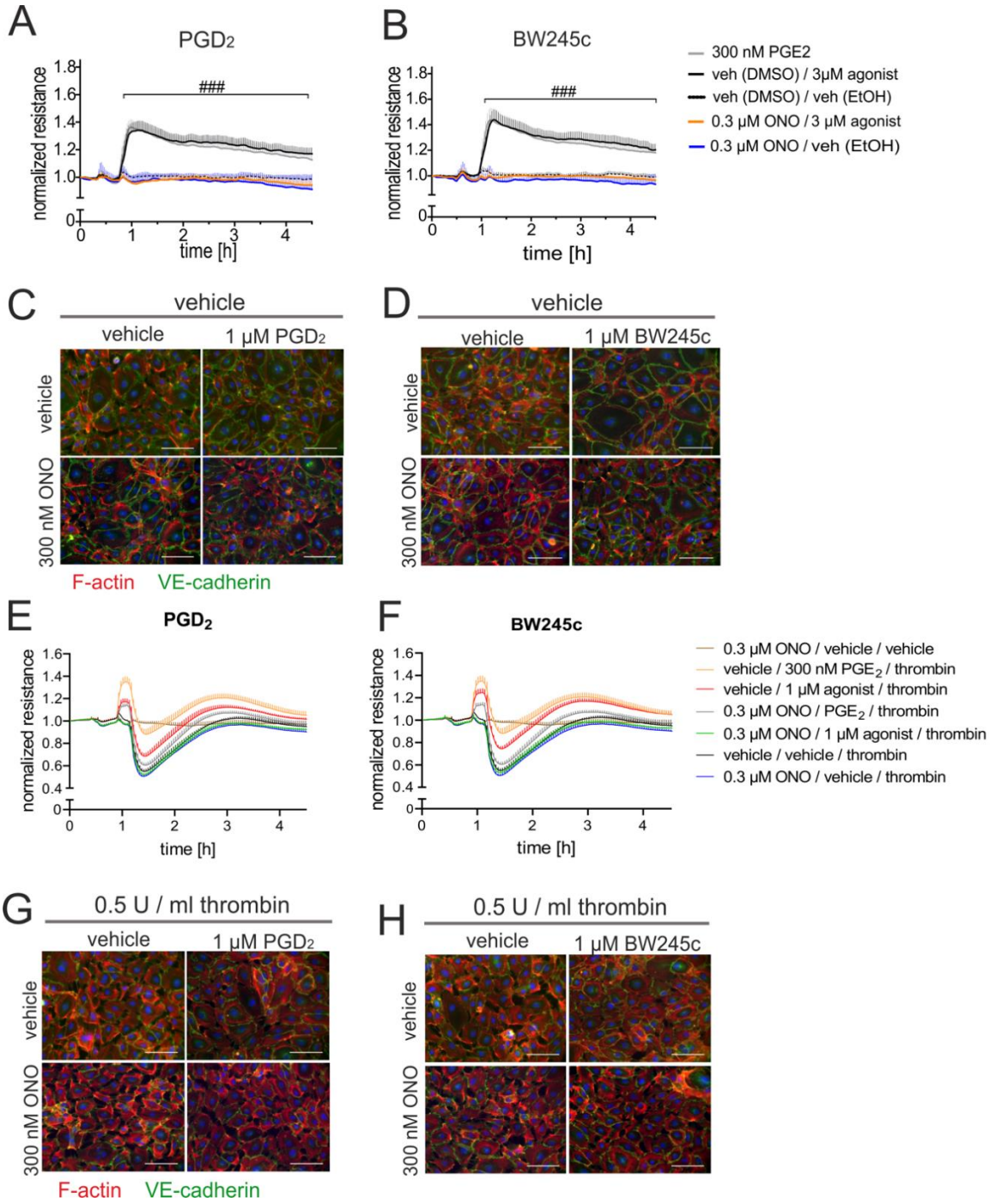

Figure 6. PGD_2 and BW245c barrier enhancement and protection against thrombin-induced barrier disruption is abolished after blockade EP4 receptors. Pre-treatment of HPMEC with 300 nM of ONO-AE3-208 (ONO; EP4 antagonist) for 30 minutes completely abolishes the barrier increase induced by 3 µM of (A) PGD_2 and (B) BW245c. VE-cadherin and F-actin staining revealed that pre-treatment with the EP4 antagonist before (C) PGD_2 or (D) BW245c stimulation inhibits VE-cadherin expression. Pre-treatment with 300 nM of ONO-AE3-208 also prevented the barrier protection against thrombin 1 µM induced by 3 µM of (E) PGD_2 and (F) BW245c. Immunofluorescent staining of VE-cadherin and F-actin showed that EP4 antagonism impairs increased VE-cadherin staining at cell periphery triggered by (G) PGD_2 or (H) BW245c in HPMEC (representative images for 5 independent experiments are shown, scale bar 100 µm). Data are displayed as mean + SEM, n = 5 - 6, two-way ANOVA for repeated measurements with Tukey's post hoc test, ### $p<0.001$ difference between veh (DMSO) / 3 µM PGD_2 or BW245c vs. antagonist / 3 µM PGD_2 or BW245c. (ONO = ONO-AE3-208)

Transient EP4 receptor knock-down in HPMEC diminishes barrier enhancement by PGD_2 and BW245c

To unequivocally rule out any non-specific effects of the EP4 antagonist, our next step was to knock-down the EP4 receptor in primary HPMEC using a siRNA approach. EP4 knock-down completely abolished the effects of PGD_2 and BW245c (Figure 7A and B, respectively). VE-cadherin and F-actin staining after transfection showed that reduction of EP4 receptor expression levels prevented PGD_2 or BW245c-induced enhancement of peripheral VE-cadherin staining (Figure 7C). RT qPCR confirmed the significantly reduced EP4 mRNA content in cells transfected with EP4-targeting siRNA (Figure 7D). Immunofluorescent staining (Figure 7E) and Western blotting (data not shown) confirmed that an incomplete knock down of EP4 receptor was achieved; however, this reduction was enough to abolish PGD_2/BW245c-mediated enhancement of barrier function. Transfection itself affected endothelial cell integrity to a certain extent as could be seen by partly decreased resistance in control siRNA-transfected HPMEC (Figure 7C). In contrast, the low levels of DP1 receptor mRNA were unchanged (Figure S9B), which was also confirmed at the protein level by immunofluorescent staining (Figure S9C). Likewise, sphingosine-1-phosphate barrier enhancement was maintained in control siRNA and EP4-targeting siRNA-treated cells (Figure S10A) as was the increase in VE-cadherin expression (Figure S10B), ruling out a toxic or non-specific effect of EP4 receptor knock-down.

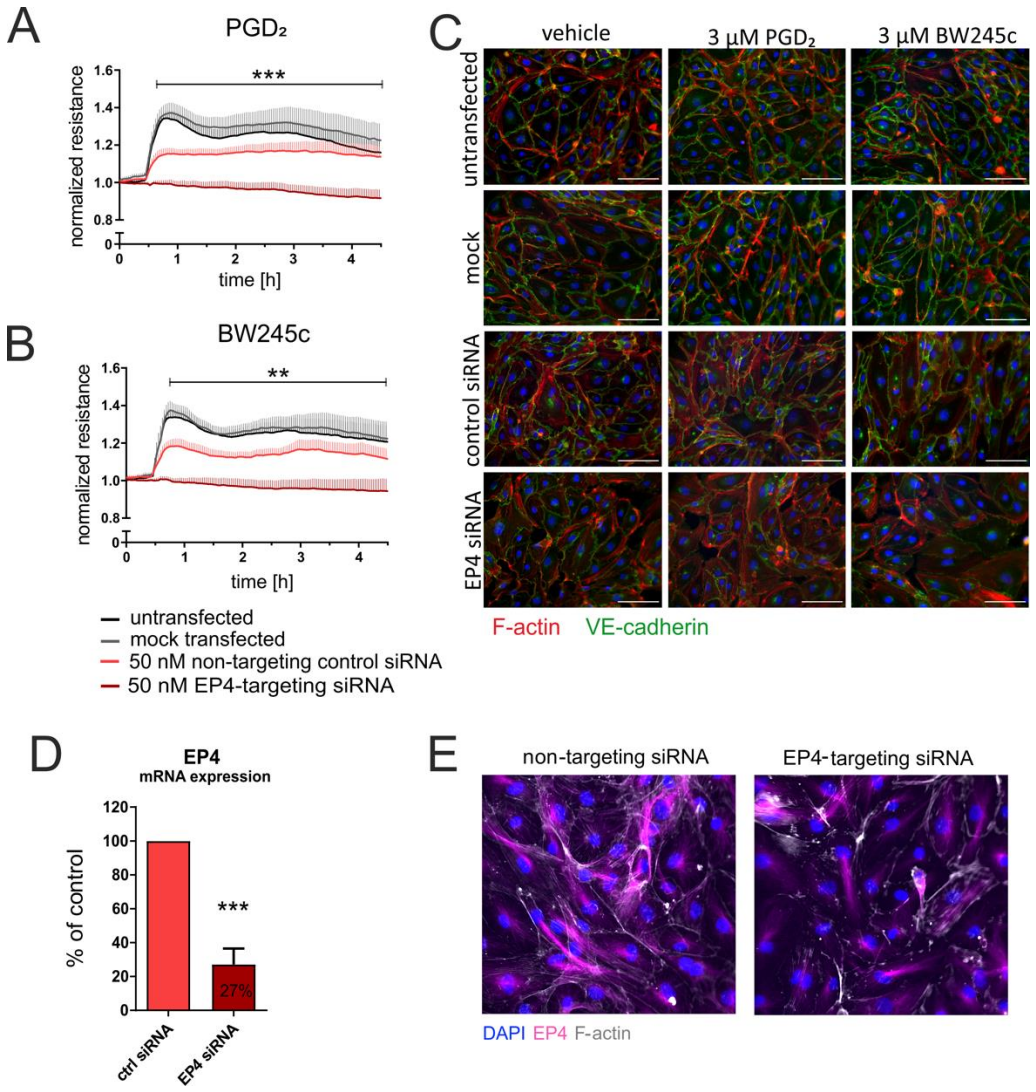

Figure 7. Transient knock-down of EP4 receptors abolished the PGD_2 and BW245c-induced HPMEC barrier enhancement. HPMECs were incubated for 48 h with transfection medium only, lipofectamin (mock transfected), non-targeting control siRNA or PTGER4-targeting siRNA (EP4-

targeting siRNA) and subsequently seeded onto 8W10E+ polycarbonate ECIS arrays for resistance measurements. EP4 knock-down completely prevented (A) PGD_2- and (B) BW245c-induced barrier enhancement. (C) VE-cadherin and F-actin staining showed that transfection with EP4-targeting siRNA caused stress fibre formation in HPMEC, without compromising the cell monolayer, while the increase in VE-cadherin at cell periphery was attenuated after stimulation with PGD_2 and BW245c. Representative images for 3 independent experiments are shown. EP4 knock-down efficiency was evaluated by (D) quantitative RT-PCR, (E) Immunofluorescence staining of EP4 receptor (representative for 3 independent experiments). Data are shown as mean + SEM, n = 6, two-way ANOVA for repeated measurements with Tukey's post hoc test or (C) Student's t-test, **p<0.01, ***p<0.001 difference between control siRNA vs. EP4 siRNA-transfected cells.

PGD_2 and BW245c promote human pulmonary artery endothelial cell barrier function through EP4 activation.

Finally, we determined whether the barrier-enhancing effect of PGD_2 and EP4 receptors can also be observed in macrovascular endothelial cells, i.e. human pulmonary artery endothelial cells (HPAEC). Indeed, PGD_2 and BW245c triggered a concentration-dependent increase also in HPAEC comparable to HPMEC (Figure 8A and B). Blockade of EP4 receptors with 300 nM ONO-AE3-208 likewise reversed barrier enhancement by both, PGD_2 and BW245c (Figure 8C and D, respectively).

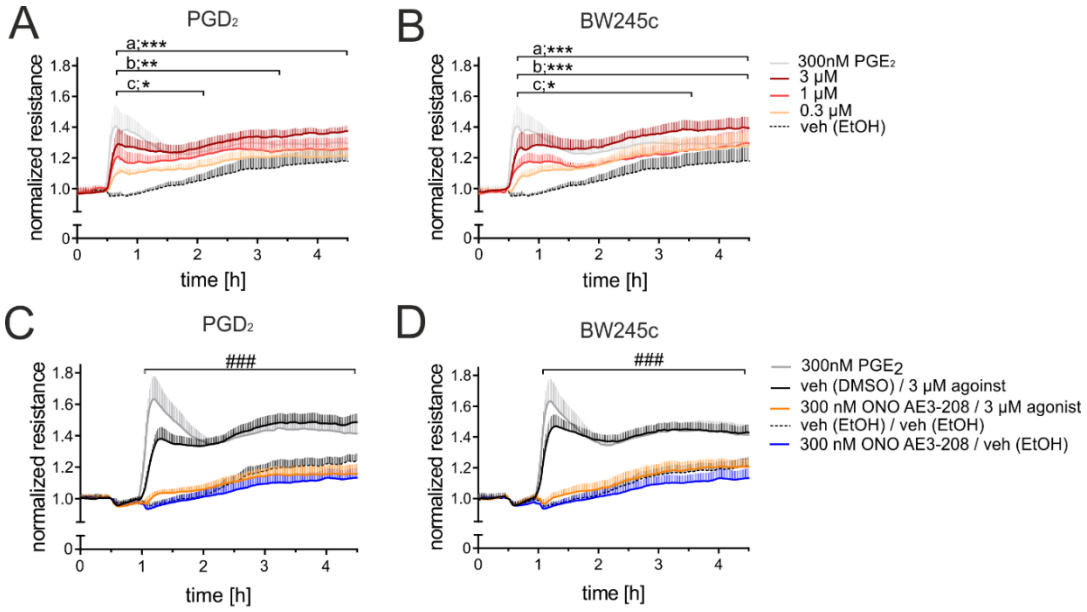

Figure 8. PGD_2 and BW245c also promote HPAEC barrier function through EP4-activation. (A) PGD_2 as well as (B) BW245c increased human pulmonary artery endothelial cell resistance in a concentration-dependent manner to a comparable level as in HPMEC. Furthermore, EP4 blockade also prevented (C) PGD_2 and (B) BW245c triggered barrier enhancement. Data are displayed as mean + SEM, n = 5. Two-way ANOVA for repeated measurements with Tukey's post hoc test, (A and B) (a) veh (EtOH) vs. 3 µM agonist, (b) veh (EtOH) vs. 1 µM agonist, (c) veh (EtOH) vs. 0.3 µM agonist, * p<0.05, ** p<0.01, *** p<0.001. (C and D) ###p<0.001 difference between veh (DMSO) / 3 µM PGD_2 or BW245c vs. 300 nM ONO-AE3-208 / 3 µM PGD_2 or BW245c.

4. Discussion

In the development of novel anti-inflammatory agents, reduction of PGD_2 production or blockade of PGD_2 signaling has been of great interest; however, PGD_2-induced signaling has proven to be more complex than previously thought. Its action depends on the target tissue, concentration and activation of different GPCRs [8,25]. Most anti-inflammatory effects of PGD_2 have been linked to DP1 receptor activation on immune, epithelial or endothelial cells [10,11,30,31]. However, in the context of pulmonary inflammation, PGD_2-DP1 activation has also been shown to induce cough in

guinea pigs [32] as well as increased mucus production and airway hyperreactivity in a mouse model of allergic asthma [33], thus making it a controversial therapeutic target. Kobayashi et al. showed that PGD_2 and the DP1 receptor agonist BW245c consistently improved human dermal microvascular endothelial cell resistance by Tiam1/Rac1-dependent cytoskeletal rearrangement [11]. Endothelial cell integrity and intact barrier function is crucial to limit leukocyte and plasma extravasation during inflammation. The aim of this study was to clarify how PGD_2 influences barrier function in human pulmonary endothelial cells.

In asthma or other acute and chronic inflammatory reactions, endothelial cell activation leading to sustained microvascular leakage is the primary cause of leukocyte and plasma extravasation [5,34]. A study using HPMEC showed that PGD_2, in comparison to other arachidonic acid-derived lipid mediators, had only a minor effect on endothelial cell barrier [35]. Here, we could show that primary HPMEC barrier integrity improved after PGD_2 or BW245c stimulation, although to a lesser extent than in HDMEC. The underlying reasons for stronger effects in HDMEC, even though HPMEC and HDMEC possess similar EP4 receptor mRNA levels, could be due to differences in intracellular signal transduction or different expression levels of barrier-associated proteins, e.g. VE-cadherin, already in steady state. In both, pulmonary and dermal microvascular endothelial cells, stimulation with PGD_2 or the DP1 agonist, BW245c but not the DP2 antagonist, DK-PGD_2 protected against thrombin-induced barrier disruption. Notably, the PGD_2-induced barrier increase in HPMEC was only partially reversed by pre-incubation with the DP1 receptor antagonist BWA868c, which was consistent with previous findings in HDMEC [11]. In the current study, a BWA868c concentration of 30 μM was required to confer a small inhibitory effect against 3 μM PGD_2 stimulation, while in a previous study a BWA868 concentration of 100 nM was sufficient to diminish inhibition of platelet aggregation by 30 nM PGD_2 [36]. Moreover, pre-treatment of cells with MK0524, an antagonist and inverse agonist of the DP1 receptor did not ablate but rather enhanced the PGD_2 effect, which largely excludes the involvement of DP1 receptors. Previously we observed that DP1 and DP2 receptors are able to form DP2/DP1 heteromers and modulate each other's signalling properties [29]. Therefore, collective blockade of both receptors might be required to inhibit downstream signalling; however, in primary human microvascular endothelial cells the combined antagonism of DP1 and DP2 receptors did not diminish the PGD_2-induced barrier enhancement.

As PGD_2 is a highly promiscuous ligand and can bind to numerous other receptors, we tested whether PPARγ or TP receptor signalling was involved. TP receptor blockade had no effect on PGD_2-induced barrier enhancement in microvascular endothelial cells. In contrast, pre-treatment with the PPARγ antagonist, T0070907, caused a transient reduction of endothelial resistance, most likely due to reduced tight junction protein expression, as previously suggested [16]. Stimulation with PGD_2 increased HPMEC resistance but the delta-increase was less than in vehicle pre-treated cells. As previously mentioned, we observed an increase in VE-cadherin staining in PGD_2-treated endothelial cells, while inhibition of PPARγ signalling might partially counteract this effect. Whether this is a direct effect of PGD_2-PPARγ interaction or as a result of PPARγ signalling interfering with junctional proteins still requires further investigation.

Throughout our experiments, we used PGE_2 as positive control and saw the same pattern of changes in trans-endothelial resistance and VE-cadherin staining, albeit to a lower extent in PGD_2/BW245c-stimulated cells. As we have previously shown that the PGE_2-mediated barrier protective effect is triggered through EP4 receptor activation in human microvascular cells [37], we hypothesized that PGD_2 might also be able to act in this fashion. This hypothesis is supported by earlier studies by Lydford et al., who indirectly concluded from pharmacological studies using rabbit saphenous vein that (1) the DP1 antagonist BWA868c has some affinity for EP4 receptors and (2) PGD_2 as well as BW245c-induced vasorelaxation was likely to be mediated through EP4 receptor activation [27]. Indeed, in our study blockade of EP4 receptors using two distinct EP4 antagonists totally reversed PGD_2-induced barrier enhancement in both, pulmonary and dermal microvascular endothelial cells, as well as in pulmonary arterial endothelial cells. The partial inhibition of barrier enhancement by the DP1 antagonist BWA868c is, therefore, most likely due to inhibition of EP4 receptor signalling. This notion is supported by two previous reports that PGD_2 may bind to, and

activate, EP4 receptors overexpressed in HEK cells with low affinity: competition in radio-ligand binding PGD_2-EP4 Ki 1483 ± 189 nM (PGE_2-EP4 Ki 0.79 ± 0.07 nM) [25] or PGD_2-EP4 mediated cAMP production EC_{50} 1664 nM (PGE_2-EP4 EC_{50} 0.3 nM) [26]. These data suggest that the affinity of PGD_2 to human EP4 receptors is approximately 1500-fold lower than of PGE2. In contrast, PGD_2 relaxed rabbit saphenous vein with 200-fold lower potency (EC50) than did PGE_2 [27], while from our study we estimate that the difference in potency with regard to TEER enhancement is near 30 fold.

Foudi et al. used immunohistochemistry as well as mRNA expression analysis in rabbit saphenous vein relaxation to reveal higher EP4 expression in human pulmonary veins in comparison to arteries [38]; however, in our experiments we could not see any obvious differences in EP4 mRNA expression levels between the different cell types, and also pulmonary artery and microvascular responses to PGD_2 and BW245c were similar. Notably, we observed very low mRNA expression of DP1 and DP2 receptors but much higher levels of EP4 receptor mRNA in primary human endothelial cells, which underlines the greater influence of EP4 receptors in endothelial cell function.

Finally, we were able to demonstrate unequivocally the involvement of the EP4 receptor in primary human microvascular cells, via EP4 receptor knock down experiments, which resulted in the total loss of barrier enhancement by PGD_2 and BW245c. Although knock down was incomplete (30 % remaining mRNA) the barrier enhancement by PGD_2 and BW245c was abolished. One explanation for this may be the rather low affinity of these ligands to EP4, such that a combination of high receptor expression levels and relatively high levels of agonist may be required for an efficient cellular response.

As mentioned above, many of the anti-inflammatory effects for PGD_2 were attributed to DP1 receptor activation. Since its discovery in 1983, BW245c has been the most commonly used DP1 agonist in cellular, preclinical and clinical studies [18,32,39–41] and its specificity has been thoroughly studied [25]. Some of the findings were confirmed using DP1 receptor or hematopoietic PGD synthase knock-out mice, in combination with BW245 treatment, which revealed very consistent, protective effects against vascular leakage, tumour angiogenesis and inflammation [10,42–44]. In mice, DP1 receptor signalling results in primarily anti-inflammatory effects; however, it is known that prostanoid receptor signalling varies vastly among different species. Certain discrepancies concerning the role of DP1 receptor activation in human and murine eosinophil function has been described [45]; DP1 receptor activation has been shown to attenuate eosinophilic inflammation in a murine model of chronic allergic lung inflammation [43] but at the same time prolongs human eosinophil survival [46]. Additionally, a study conducted in 2011 revealed that PGE_2 induced relaxation by activation of EP2 receptor in mice, guinea pigs and monkey trachea, while in human and rat the relaxation is EP4 receptor mediated [47]. In this study, we could show that EP4, but not DP1, is the primary receptor responsible for PGD_2-induced improvement of barrier function in human endothelial cells.

In the context of severe pulmonary inflammatory responses, such as acute lung injury or exacerbations, it may be speculated that PGD_2-EP4-induced enhancement of endothelial barrier plays a crucial role in limiting edema formation. Bacterial products i.e. lipopolysaccharide (LPS) have been shown to induce PGD_2 production in murine bone marrow-derived macrophages [48], which may be able to influence inflammation in return. Interestingly, in a low-dose LPS model of acute lung injury, PGD_2 exacerbated pulmonary inflammation in mice [49], while another group showed that PGD_2 limited inflammation in the same model using a higher LPS dose [10]. One possible explanation for this could be that high levels of PGD_2 trigger the physiologic response to limit inflammation by strengthening the endothelial barrier, thereby limiting edema formation and the risk of sepsis. In allergic asthma, mast cell-derived PGD_2 recruits eosinophils and Th2 cells via DP2 activation [50], while PGD_2 levels from other sources, e.g. monocytes/macrophages, might activate EP4 receptors on endothelial cells and prevent excess edema formation.

5. Conclusion

We here demonstrate for the first time that PGD_2 enhances the barrier function of human pulmonary and dermal endothelial cells by activating EP4 receptors, but independently from

classical PGD_2 receptors, DP1 and DP2, as well as TP receptors. These findings thus reveal yet another way of how PGD_2 might modulate inflammation and raises the question, whether some of the anti-inflammatory effects observed with PGD_2 might be due to EP4 activation. Moreover, we could further substantiate the potential of EP4 receptor activation as therapeutic approach to counteract vascular leakage.

Supplementary Materials: Figure S1. PGD_2 and DP_1 agonist BW245c but not DP2 agonist DK-PGD_2 protect HDMECs against thrombin-induced barrier disruption. **Figure S2.** DP1 antagonist MK0524 does not block PGD_2-induced barrier enhancement in HPMEC. **Figure S3.** DP1 and DP2 receptor antagonists do not prevent the PGD_2-induced barrier increase in HDMEC. **Figure S4**. EP4, DP1, DP2 receptor mRNA expression levels in HDMEC, HPAEC and human peripheral blood monocytes, and EP4 / DP1 immunofluorescence staining in HPMEC. **Figure S5.** EP4 antagonism prevents PGD_2 and BW245c barrier enhancement and protection against thrombin-induced barrier disruption in HDMEC. **Figure S6.** EP4 antagonist GW627368X strongly attenuates the barrier-protective effect of PGD_2 and BW245c**. Figure S7.** ONO-AE3-208 does not inhibit sphingosine-1-phosphate-induced barrier enhancement in HPMEC**. Figure S8.** ONO-AE3-208 does not affect PGD_2-induced inhibition of platelet aggregation**. Figure S9.** Transient knock-down of EP4 receptor does not affect DP1 receptor expression in HPMEC. **Figure S10.** Transient knock-down of EP4 receptors does not affect the early phase of sphingosine-1-phosphate barrier enhancement.

Author Contributions: SR designed and performed experiments, analyzed data, interpreted the results, wrote and edited the manuscript. KR, WP, EK, NS, RA and CN performed experiments and analyzed data. AH designed experiments, interpreted the results, supervised the study, wrote and edited the manuscript. All authors have read and agree to the published version of the manuscript.

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