

Endothelial E-type prostanoid 4 receptors promote barrier function and inhibit neutrophil trafficking

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Background: Increased vascular permeability is a fundamental characteristic of inflammation. Substances that are released during inflammation, such as prostaglandin (PG) E₂, can counteract vascular leakage, thereby hampering tissue damage. **Objective:** In this study we investigated the role of PGE₂ and its receptors in the barrier function of human pulmonary microvascular endothelial cells and in neutrophil trafficking. **Methods:** Endothelial barrier function was determined based on electrical impedance measurements. Neutrophil recruitment was assessed based on adhesion and transendothelial migration. Morphologic alterations are shown by using immunofluorescence microscopy.

Results: We observed that activation of E-type prostanoid (EP) 4 receptor by PGE₂ or an EP4-selective agonist (ONO AE1-329) enhanced the barrier function of human microvascular lung endothelial cells. EP4 receptor activation prompted similar responses in pulmonary artery and coronary artery endothelial cells. These effects were reversed by an EP4 antagonist (ONO AE3-208), as well as by blocking actin polymerization with cytochalasin B. The EP4 receptor-induced increase in barrier function was independent of the classical cyclic AMP/protein kinase A signaling machinery, endothelial nitric oxide synthase, and Rac1. Most importantly, EP4 receptor stimulation showed potent anti-inflammatory activities by (1) facilitating wound healing of pulmonary microvascular endothelial monolayers, (2) preventing junctional and cytoskeletal reorganization of activated endothelial cells, and (3) impairing neutrophil adhesion to endothelial cells and transendothelial migration. The latter effects could be partially attributed to reduced E-selectin expression after EP4 receptor stimulation.

Conclusion: These data indicate that EP4 agonists as anti-inflammatory agents represent a potential therapy for diseases with increased vascular permeability and neutrophil extravasation. (J Allergy Clin Immunol 2012;■■■■:■■■■-■■■■.)

Key words: Chronic obstructive pulmonary disease, acute lung injury, endothelial barrier, prostaglandins, vascular leakage, neutrophil trafficking

Under inflammatory conditions, dysfunction of the vascular endothelial barrier leads to increased permeability of the vasculature.¹ The integrity of the endothelial barrier is controlled by interendothelial cell contacts, such as tight and adherens junctions, and by connection of endothelial cells with the extracellular matrix through focal adhesion points.^{2,3} Regulation of the endothelial barrier is dependent on the association of these multiprotein complex sites to the actin cytoskeleton.⁴ Sustained increase in vascular permeability causes edema and facilitates inflammatory responses and leukocyte recruitment to inflammatory sites.⁵ In the lung the microvascular endothelial cells situated on a thin basement membrane form a complete barrier between the blood and the interstitium.⁶ Inflammatory conditions, such as sepsis and acute lung injury, severely compromise the microvasculature, and endothelial barrier-promoting agents, such as sphingosine-1-phosphate (S1P) and simvastatin, have recently been suggested as possible pharmacologic therapies in these pathophysiologic settings.⁷

Prostaglandin (PG) I₂ and PGE₂ have been shown to increase endothelial barrier function.⁸ PGE₂ is a major COX-derived product of fibroblasts, monocytes, and epithelial and endothelial cells and is abundant in inflammation.⁹ PGE₂ is involved in various biological processes, including pain, fever, inflammation, angiogenesis, and tumorigenesis, and often shows opposing effects that might be due to its affinity to 4 so-called E-type prostanoid (EPs) receptors.¹⁰ These receptors are coupled to different G proteins and accordingly activate diverse signaling pathways: EP1 couples to G_{αq} and increases intracellular Ca²⁺ levels, EP2 and EP4 are G_{αs} coupled and induce cyclic AMP (cAMP) production, and EP3 binds to G_{αi} and inhibits cAMP synthesis.¹¹

Here we set out to investigate the role of EP4 receptors in the regulation of endothelial barrier function. We describe that EP4 receptor activation increases endothelial electrical resistance and adherens junctions and prevents the interaction of neutrophils with pulmonary endothelial cells, such as adhesion under flow conditions and transendothelial migration. Our data suggest an anti-inflammatory role of EP4 receptors in the pulmonary circulation.

METHODS

Culture of endothelial cells

Human lung microvascular endothelial cells (HMEC-Ls), human pulmonary artery endothelial cells (HPAECs), and human coronary artery

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Abbreviations used

cAMP:	Cyclic adenosine monophosphate
ECIS:	Electric Cell-substrate Impedance Sensing system
EP:	E-type prostanoid
F-actin:	Filamentous actin
HCAEC:	Human coronary artery endothelial cell
HMVEC-L:	Human lung microvascular endothelial cell
HPAEC:	Human pulmonary artery endothelial cell
PG:	Prostaglandin
PI3K:	Phosphoinositide 3-kinase
PKA:	Protein kinase A
PKC:	Protein kinase C
S1P:	Sphingosine-1-phosphate

endothelial cells (HCAECs) were purchased from Lonza (Verviers, Belgium). HMVEC-Ls and HCAECs were maintained in EGM-2 MV BulletKit medium (Lonza) with 5% FCS. HPAECs were maintained in EGM-2 BulletKit medium with 2.5% FCS. Endothelial cells were cultivated as previously described.^{12,13} For further details on reagents, see the **Methods** section in this article's Online Repository at www.jacionline.org.

Preparation of human peripheral blood neutrophils

Blood sampling from healthy volunteers who were not taking any medication was approved by the Institutional Review Board of the Medical University of Graz. Polymorphonuclear leukocytes (containing eosinophils and neutrophils) were purified as previously described.^{14,15} Purified polymorphonuclear leukocytes showed greater than 97% purity and viability.

Endothelial electrical impedance and wound-healing measurements

Endothelial cells (1.25×10^5 per chamber) were grown to confluence on 1% gelatin-precoated 8W10E+ polycarbonate arrays containing gold micro-electrodes (Applied Biophysics, Troy, NY). Cells were serum starved for 1 hour before performing impedance measurements at multiple frequencies by using the Electric Cell-substrate Impedance Sensing (ECIS) system (Applied Biophysics, Troy, NY).^{16,17}

Cells were grown on ECIS 8W1E bioarrays to study the regenerative properties of endothelial monolayers. Confluent layers of endothelial cells were electrically wounded (5 seconds at 900 μ A and 48 kHz), resulting in severe electroporation and subsequent death of the cells situated on the electrodes. Impedance was continuously monitored to detect repopulation of the wound.¹⁸

Neutrophil adhesion

Endothelial cells (3.5×10^4 per well) grown into confluent cell layers in 48-well plates were preincubated with agonists for 10 minutes, followed by incubation with TNF- α for 4 hours. Purified human neutrophils (6×10^4 per well) were then incubated with endothelial layers in the absence or presence of IL-8 for 30 minutes at 37°C.¹² Nonadherent and adherent neutrophils were counted by using flow cytometry. Neutrophil adhesion was expressed as a percentage of adherent cells of totally added cells.

To study neutrophil adhesion under flow conditions, we plated endothelial cells (4×10^5 cells) on VenaEC biochips (Cellix, Dublin, Ireland). Confluent endothelial layers were pretreated with agonists for 10 minutes, followed by incubation with TNF- α for 4 hours. Neutrophils were preconditioned in EBM-2 MV medium for 5 minutes at 37°C. Endothelial monolayers were superfused with suspensions of 3×10^6 /mL neutrophils in EBM-2 MV medium at 0.5 dyne/cm² for 2 minutes and 20 seconds at 37°C. Cell adhesion was monitored by using phase contrast microscopy.^{19,20}

Neutrophil transendothelial migration

Endothelial cells (3.5×10^4) were grown to confluence in 5- μ m filters in 6.5-mm Transwell inserts (Corning, Corning, NY). The monolayers treated as indicated were placed into 24-well plates containing medium or IL-8 in the basolateral compartment, and 2×10^5 neutrophils were added apically to the endothelial cells. The plates were incubated at 37°C in a humidified incubator for 3 hours, and the nonmigrated and transmigrated neutrophils were separately enumerated by using flow cytometry. Neutrophil transmigration was calculated as a percentage of transmigrated neutrophils on the basis of total cells added.¹⁹

Statistics

All data are shown as means + SEMs for *n* observations. Statistical analyses were performed by using SigmaPlot 12 software (Systat Software, Inc, San Jose, Calif) with 1-way repeated-measurement ANOVA with the Bonferroni *post hoc* test. *P* values of less than .05 were considered statistically significant.

RESULTS**Stimulation of EP4 receptors enhances endothelial barrier function**

The selective EP4 agonist ONO AE1-329 (ONO Pharmaceutical, Osaka, Japan) and PGE₂ concentration-dependently (10 – 300 nmol/L) increased endothelial barrier function, which was recorded as electrical impedance, in HMVEC-Ls and, to a lesser extent, in macrovascular HPAECs (Fig 1, A and B). In contrast, the barrier-enhancing effect of S1P (Fig 1, C) was less pronounced in HMVEC-Ls compared with that seen in HPAECs. Specific involvement of the EP4 receptor was demonstrated by preincubating the monolayers with the EP4-selective antagonist ONO AE3-208 (300 nmol/L). Endothelial barrier enhancement was induced by EP4 agonist and PGE₂ (30 nmol/L), and this was completely prevented by the EP4 antagonist (Fig 2, A). Similar observations were made in HCAECs, which were used as a nonpulmonary endothelial cell model (Fig 2, A).

The expression of EP4 receptor on HMVEC-Ls was assessed by using flow cytometry and Western blotting with an EP4-specific mAb. Original histograms show high expression of EP4 receptors, and a single band was detected in Western blots at an apparent molecular size of 52 kDa in 3 different lysates of HMVEC-Ls (Fig 2, B, and see the **Methods** section in this article's Online Repository).

Actin polymerization, but not cAMP, mediates the EP4-induced endothelial barrier enhancement

Endothelial barrier functionality has been proposed to correlate with alterations of the cytoskeletal network, actin filaments, and microtubules.^{4,21} In fact, inhibition of actin polymerization with cytochalasin B (2 μ g/mL) and disruption of microtubules with colchicine (1 μ mol/L) for 1 hour profoundly compromised the impedance of the monolayers. Cytochalasin B, but not colchicine, prevented the barrier-enhancing effect of the EP4 agonist and S1P in HMVEC-Ls, as well as in HPAECs (Fig 3). These observations suggest that EP4 receptors reinforce endothelial barrier function through actin-mediated but not microtubule-mediated events.

Being a G α_s protein-coupled receptor, stimulation of the EP4 receptor leads to an increase in intracellular cAMP levels.¹¹ EP4 stimulation of HMVEC-Ls with ONO AE1-329 (300 nmol/

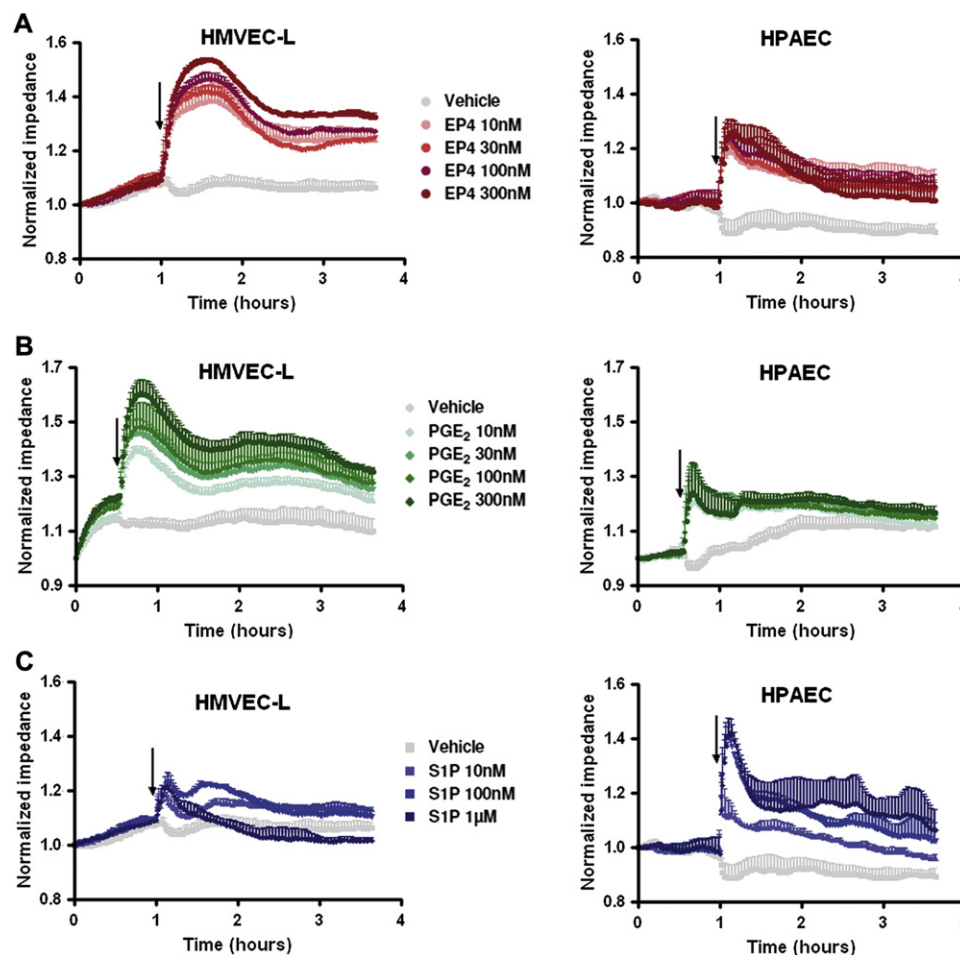


FIG 1. EP4 receptors increase the barrier function of pulmonary microvascular and macrovascular endothelial cell layers. HMVEC-Ls and HPAECs were treated with the EP4-selective agonist ONO AE1-329 (A), PGE₂ (B), S1P (C) or vehicle (arrows). Data show normalized impedance means + SEMs of 3 independent experiments.

L) caused a small increase in cAMP production, which was significantly enhanced after pretreatment of the cells with 0.5 mmol/L isobutylmethylxanthine for 10 minutes to block phosphodiesterase activity (Fig 4, A). Incubation with PGE₂ (300 nmol/L) did not significantly increase cAMP levels, whereas forskolin (20 μ mol/L), an adenylyl cyclase activator, markedly enhanced cAMP production by HMVEC-Ls (Fig 4, A, and see the **Methods** section in this article's Online Repository). Inhibitors of adenylyl cyclase (SQ22356, 10 μ mol/L) and protein kinase A (PKA; H-89, 10 μ mol/L) were applied to assess the contribution of the cAMP pathway in the endothelial barrier-enhancing function of the EP4 receptor (Fig 4, B). Importantly, preincubation of the endothelial monolayers with the inhibitors for 1 hour did not prevent the EP4 agonist-induced increase in electrical impedance (Fig 4, B). This correlated well with the observation that PGE₂ did not increase cAMP levels (Fig 4, A) but still enhanced endothelial barrier function (Figs 1 and 2).

In further experiments we probed the involvement of additional signaling pathways using respective inhibitors of Rac1 (NSC 23766, 100 μ mol/L), endothelial nitric oxide synthase (L-NAME, 100 μ mol/L), phosphoinositide 3-kinase (PI3K; LY294002, 10 μ mol/L), and protein kinase C (PKC;

chelerythrine, 1 μ mol/L). However, none of these inhibitors reversed the barrier-promoting effect of the EP4 agonist (see Fig E1 in this article's Online Repository at www.jacionline.org). These data suggested that the EP4 receptor-induced barrier enhancement is regulated by reorganization of the actin cytoskeleton and is independent of the cAMP pathway.

Activation of EP4 receptors accelerates endothelial wound healing and protects junctional integrity

The involvement of EP4 receptors in wound healing of endothelial cell layers was investigated by using an electrical wounding approach.¹⁸ The EP4-selective agonist and PGE₂ (30 nmol/L each) facilitated the recovery of electrically wounded HMVEC-L layers, which was comparable with the effect of vascular endothelial growth factor (50 ng/mL, Fig 5). In addition to PGE₂ and the EP4-selective agonist, other EP agonists, namely butaprost (EP2 agonist, 30 nmol/L) and sulprostone (EP1/3 agonist, 30 nmol/L), were tested (Fig 5). We found that pulmonary microvascular and macrovascular endothelial cells express EP1, EP3, and EP4 receptors to a similar extent, whereas they lack EP2 receptors (data not shown). Stimulation of EP1/3 receptors

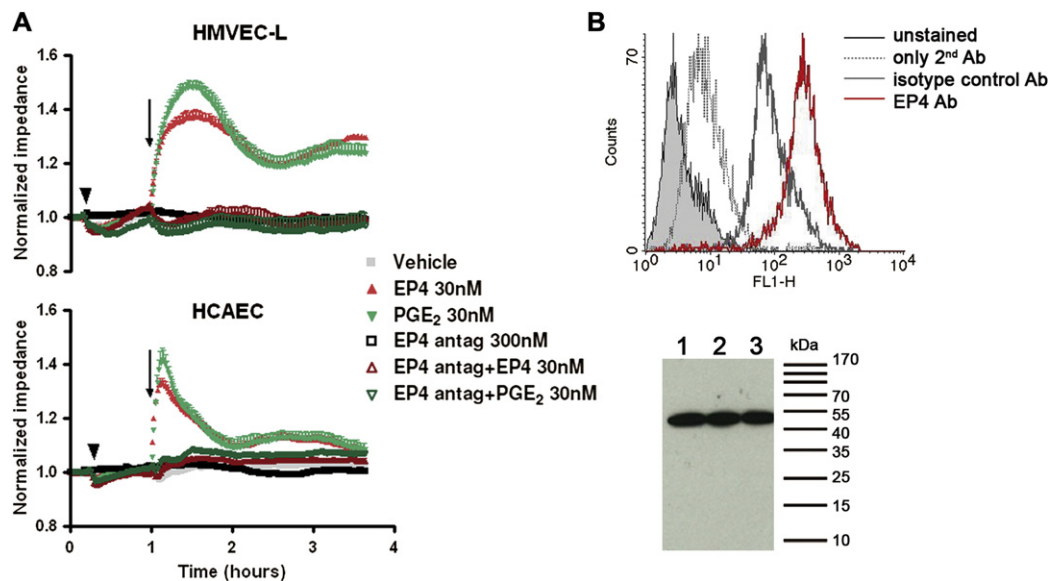


FIG 2. The PGE₂-induced increase in endothelial barrier function is mediated by EP4 receptors. **A**, HMVEC-Ls and HCAECs were preincubated with the EP4-selective antagonist ONO AE3-208 or vehicle (arrowheads), followed by ONO AE1-329 (EP4) or PGE₂ (arrows). Data show normalized impedance means + SEMs (n = 3). **B**, EP4 expression on HMVEC-Ls is demonstrated by using flow cytometry and Western blotting (n = 3). Ab, Antibody.

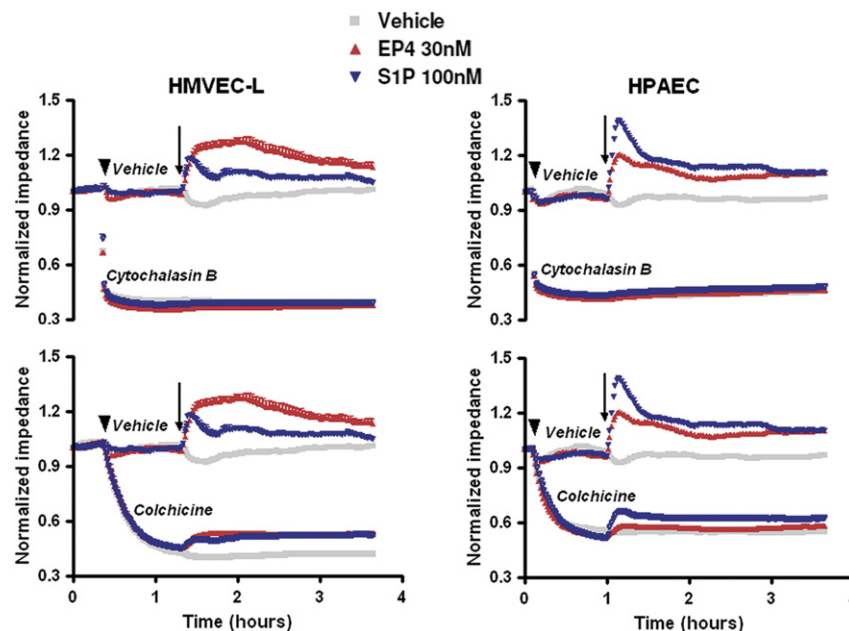


FIG 3. Cytoskeletal regulation of the EP4-induced enhancement of endothelial barrier function. HMVEC-Ls and HPAECs were pretreated with cytochalasin B or colchicine (arrowheads) and then stimulated with the EP4 agonist ONO AE1-329, S1P, or vehicle (arrows). Data show normalized impedance means + SEMs (n = 3).

by sulprostone and EP2 receptors by butaprost, however, did not facilitate healing of electrically wounded endothelial cell layers. In contrast, antagonizing EP4 receptors with ONO AE3-208 (300 nmol/L) for 15 minutes almost completely reversed the PGE₂-enhanced wound healing (Fig 5).

Endothelial junctional organization was visualized by means of immunofluorescent staining of VE-cadherin, the main component

of adherens junctions.² Adherens junctions of confluent endothelial monolayers appeared as condensed structures at the cell periphery between adjacent cells (Fig 6, A). Stimulation of EP4 receptors with the EP4 agonist or PGE₂ (30 nmol/L) for 10 minutes resulted in more intense VE-cadherin staining in the junctional zone (Fig 6, A) and induced cortical actin formation (Fig 6, B). The junction-enhancing effect of PGE₂ was reversed by means of pretreatment

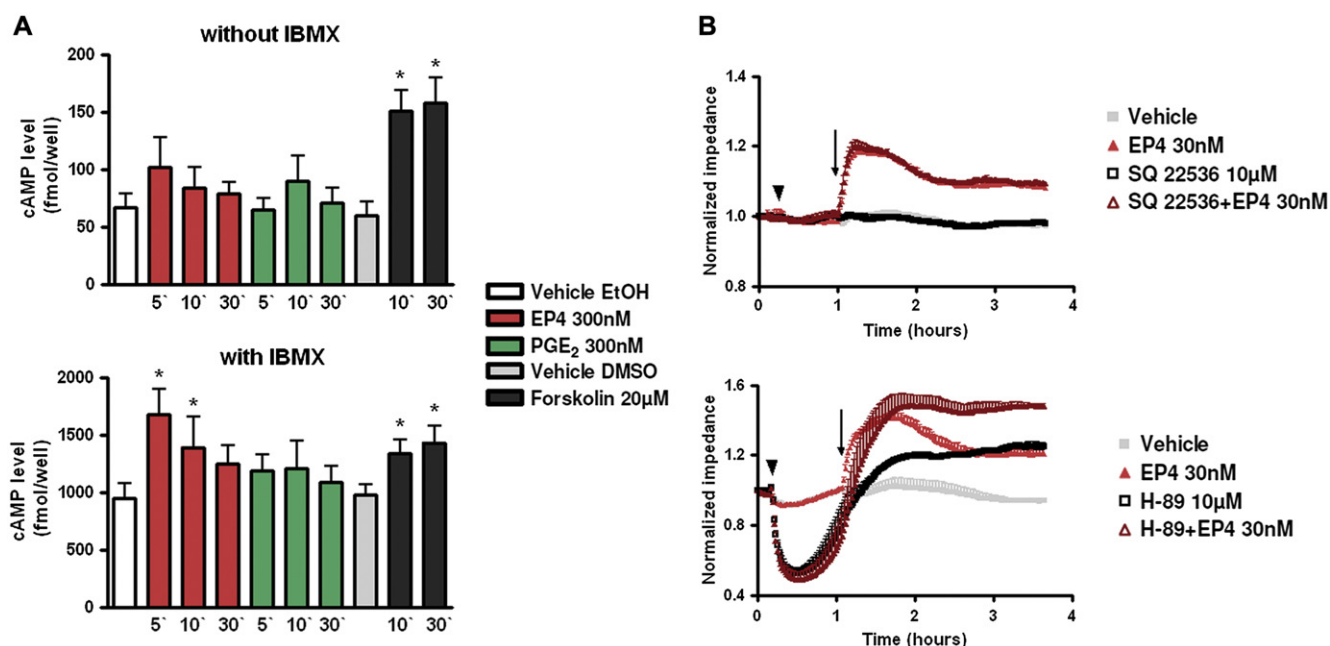


FIG 4. cAMP does not regulate the EP4-induced barrier enhancement. **A**, HMVEC-Ls were stimulated with ONO AE1-329 (EP4), PGE₂, or forskolin in the absence or presence of isobutylmethylxanthine (IBMX). Data show mean + SEM total cAMP content (n = 3). *P < .05 versus vehicle ethanol. **B**, HMVEC-Ls were pre-treated with the adenylyl cyclase inhibitor SQ22536 or the PKA inhibitor H-89 (arrowheads) and stimulated with ONO AE1-329 or its vehicle (arrows). Data show normalized impedance means + SEMs (n = 3).

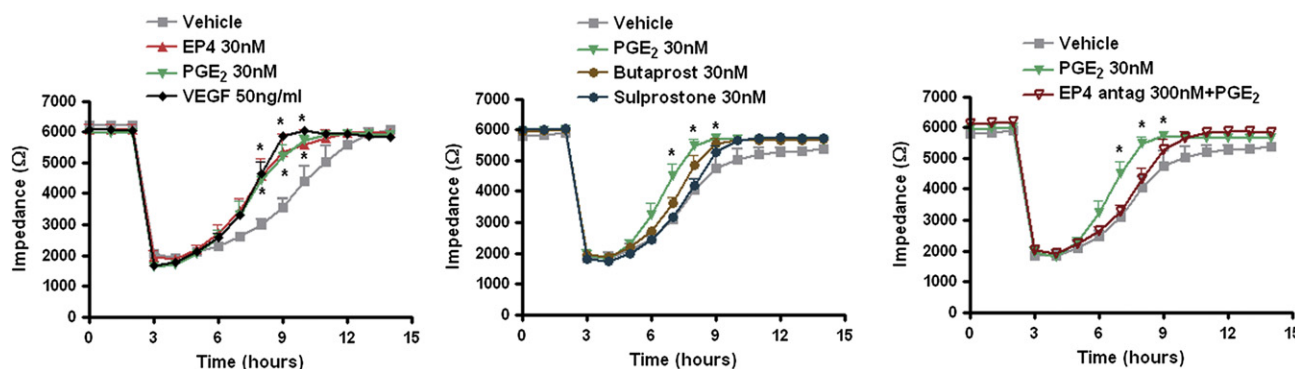


FIG 5. The EP4 agonist and PGE₂ stimulate wound healing of endothelial cells. HMVEC-Ls were treated with ONO AE1-329 (EP4), PGE₂, butaprost, sulprostone, or vascular endothelial growth factor and, in some experiments, with the EP4 selective antagonist ONO AE3-208, followed by electric wounding. Data show mean + SEM impedance (Ω; n = 3). *P < .05 versus vehicle.

with the EP4 antagonist ONO AE3-208 for 15 minutes (Fig 6, A). To mimic the inflammation-induced alterations of endothelial barrier structure, we exposed endothelial layers to thrombin (0.5 U/mL) for 15 minutes, which led to stress-fiber formation and junctional rearrangement (ie, gap formation between neighboring cells; Fig 6, A and B). Importantly, thrombin-induced intercellular gap formation and induction of stress fibers were prevented by means of preincubation of HMVEC-Ls with the EP4 agonist and PGE₂ (Fig 6, A and B). Specific involvement of EP4 receptors was demonstrated by applying the EP4 antagonist 15 minutes before incubation with PGE₂, which resulted in contracted endothelial cells with abolished junctional networks (Fig 6, A and B). Quantitation of VE-cadherin expression in the junctional zone and cortical filamentous actin (F-actin) is shown in Fig 6, C (see the Methods

section in this article's Online Repository). In summary, EP4 receptor activation promotes the repair of endothelial monolayers and protects against junctional disruption.

Stimulation of EP4 receptors reduces neutrophil trafficking across the endothelium

Only few neutrophils, less than 10% of input cells, adhered to resting endothelial cells after 30 minutes (Fig 7, A). In contrast, preincubation of endothelial cells with TNF-α (5 pmol/L) for 4 hours induced adhesion of 50% of neutrophils, and this was further enhanced when neutrophils were exposed to increasing concentrations of IL-8 (0.3-3 nmol/L) for 10 minutes before being added to the endothelial cells. Preincubation of endothelial cells

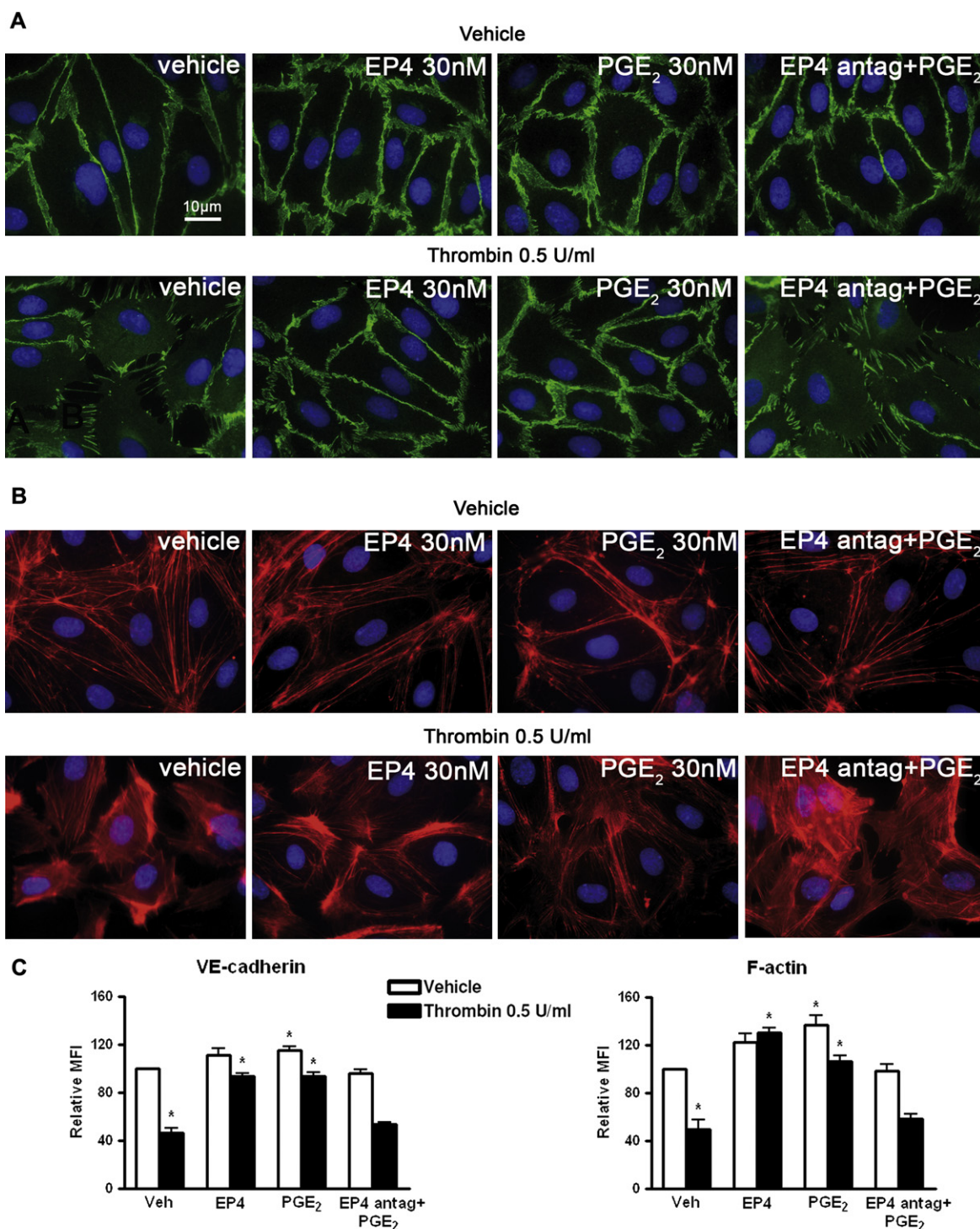


FIG 6. EP4 receptor protects against thrombin-induced disruption of endothelial junctions. HMVEC-Ls treated with ONO AE1-329 (EP4) or PGE₂ or preincubated with 300 nmol/L of the EP4 antagonist ONO AE3-208 were exposed to vehicle or thrombin. **A** and **B**, Immunofluorescence micrographs show staining with anti-VE-cadherin antibody (green; Fig 6, **A**) and Texas Red-conjugated phalloidin (red; Fig 6, **B**) and 4'-6-diamidino-2-phenylindole dihydrochloride (blue). **C**, Mean fluorescence intensity (MFI) + SEM relative to control treatment (n = 3). *P < .05 versus vehicle/thrombin.

with the EP4 agonist for 10 minutes slightly reduced the adhesion of neutrophils to resting endothelial cells and significantly inhibited the TNF- α -induced neutrophil adhesion by 30% to 45% both in the absence and presence of IL-8 (Fig 7, **A**). Similar

observations were made under physiologic flow conditions, during which neutrophil adhesion was recorded for a period of 140 seconds. Either neutrophils were pretreated with IL-8 (3 nmol/L) for 5 minutes or endothelial cells were pretreated

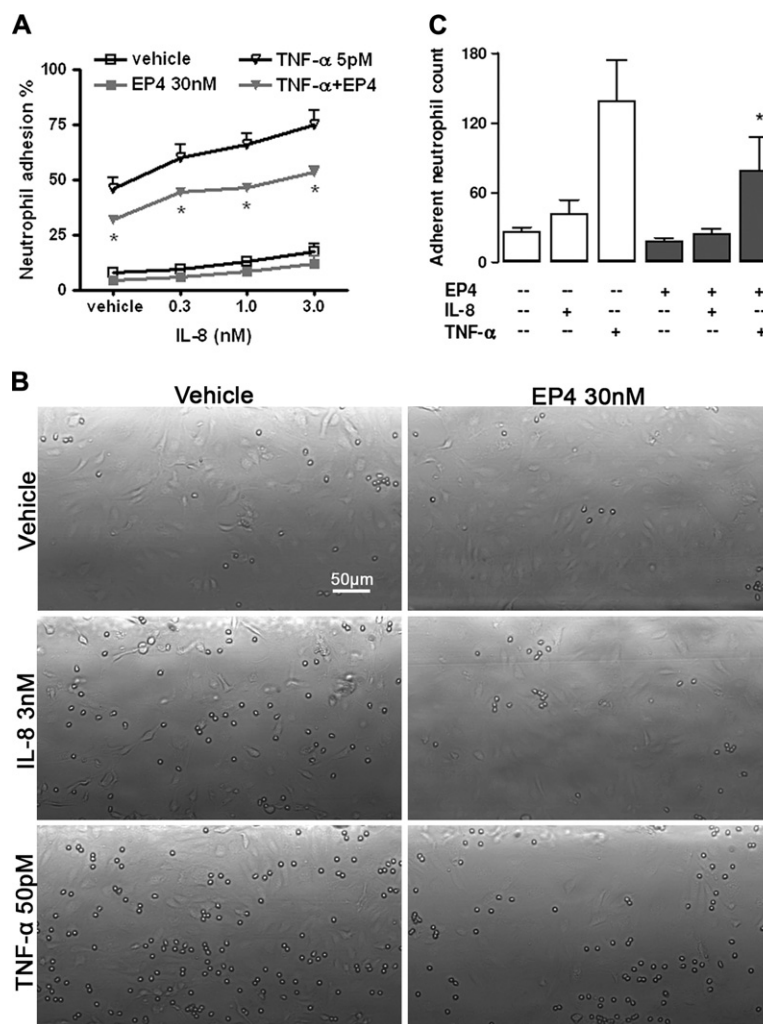


FIG 7. EP4 agonist hinders neutrophil adhesion to endothelial cells. **A**, Endothelial monolayers were pretreated with vehicle, ONO AE1-329 (EP4), TNF- α , or both. Neutrophil adhesion stimulated with IL-8 is shown as mean + SEM percentages ($n = 4$). * $P < .05$, TNF- α plus EP4 agonist versus TNF- α alone. **B** and **C**, Neutrophils were superperfused over endothelial cells pretreated with EP4 agonist, TNF- α , or both. In some experiments neutrophils were stimulated with IL-8. In Fig 7, **B**, representative images were taken after 2 minutes. Fig 7, **C**, shows quantitative data of neutrophil adhesion as means + SEMs ($n = 4$). * $P < .05$, EP4 agonist plus TNF- α versus TNF- α alone.

with TNF- α (50 pmol/L) for 4 hours to stimulate adhesion. On average, 25 neutrophils per high-power field became adherent spontaneously. In contrast, approximately 45 neutrophils adhered to endothelial monolayers after IL-8 stimulation and more than 120 neutrophils adhered to endothelial monolayers after TNF- α activation of endothelial cells (Fig 7, **B** and **C**). Pretreatment of endothelial cells with the EP4 agonist (30 nmol/L) allowed significantly less neutrophils to become adherent (Fig 7, **B**). Representative video footage can be found as [Video E1](#) in this article's Online Repository at www.jacionline.org.

E-selectin mediates the capture and adhesion of leukocytes to endothelial cells.²² Therefore we investigated the effect of EP4 receptor activation on endothelial E-selectin expression. Treatment of endothelial cells with TNF- α (50 pmol/L) for 4 hours resulted in upregulation of E-selectin by a factor of 8.8 ± 2.8 (data not shown). This effect was reduced by the EP4 agonist and PGE₂ (30 nmol/L) applied 10 minutes before TNF- α , decreasing E-selectin expression to 65% to 70% (Fig 8, **A**).

To identify the underlying signaling mechanism, we used several inhibitors of relevant signaling pathways to pretreat the endothelial cells for 30 minutes before EP4 agonist and PGE₂. Inhibition of the adenylyl cyclase activity with SQ22356 (10 μ mol/L) did not reverse the effects of the EP4 agonist and PGE₂ to reduce E-selectin expression. In contrast, inhibition of PKC (chelerythrine, 1 μ mol/L) and PI3K (LY294002, 1 μ mol/L) almost completely reversed the EP4 agonist- and PGE₂-induced decrease in E-selectin expression. Inhibition of extracellular signal-regulated kinase 1/2 with PD184161 (1 μ mol/L) had little effect, whereas the selective EP4 antagonist ONO AE3-208 (300 nmol/L) completely reversed both the EP4 agonist- and PGE₂-induced decrease in E-selectin expression (see the [Methods](#) section in this article's Online Repository).

The role of EP4 receptor in controlling neutrophil trans-endothelial trafficking was studied in Transwell plates for a period of 3 hours (Fig 8, **B**). Neutrophil transendothelial migration was triggered by IL-8 (3 nmol/L) placed in the bottom wells.

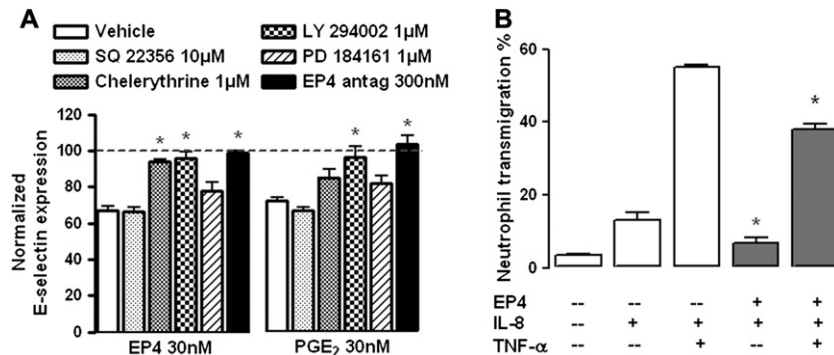


FIG 8. EP4 receptor blocks TNF- α -induced upregulation of endothelial E-selectin and reduces neutrophil transendothelial migration. **A**, HMVEC-Ls pretreated with inhibitors were treated with the EP4 agonist ONO AE1-329 or PGE₂. TNF- α -induced E-selectin expression was detected by means of flow cytometry and expressed as mean + SEM percentage of control (n = 6-8). **P* < .05 versus EP4 agonist or PGE₂ alone. **B**, Endothelial cells in Transwell inserts were pretreated with EP4 agonist, TNF- α , or both. Percentages of transmigrated neutrophils toward IL-8 are shown as means + SEMs (n = 4). **P* < .05 versus IL-8 or TNF- α alone.

In some experiments the confluent endothelial monolayers were additionally activated by TNF- α (50 pmol/L for 4 hours). IL-8 caused 15% of input neutrophils to transmigrate, whereas 55% transmigration was observed in combination with TNF- α (Fig 8, B). EP4 agonist pretreatment of endothelial monolayers for 10 minutes significantly reduced the IL-8-induced transmigration to 7% and the IL-8/TNF- α -triggered transmigration to less than 40% of input cells. Collectively, EP4 receptors negatively regulate the interaction of neutrophils with endothelial cells by decreasing E-selectin expression on endothelial cells.

DISCUSSION

Our results demonstrate that activation of the PGE₂ receptor EP4 increases basal barrier function and prevents barrier dysfunction induced by edemagenic stimuli. We found that the EP4-selective agonist ONO AE1-329 and PGE₂ itself concentration-dependently increased the barrier function of human pulmonary microvascular and macrovascular endothelial cells, as indicated by an increase in electrical impedance. The effects of both PGE₂ and the EP4 agonist were completely prevented by the selective EP4 antagonist ONO AE3-208. Additionally, we showed that HCAECs responded to PGE₂ and EP4 receptor agonist with barrier increase similar to pulmonary endothelial cells. These findings argue for a general barrier-promoting effect of EP4 receptors ranging from the pulmonary endothelium to the vasculature of other organs.

Barrier-enhancing substances, such as S1P, facilitate rapid polymerization of F-actin, resulting in cortical actin enhancement.¹ Disruption of actin polymerization with cytochalasin B entirely blocked the S1P-induced barrier increase,²³ pointing to a crucial role of the actin cytoskeleton. Similarly, the EP4 receptor-induced barrier enhancement in pulmonary microvascular endothelial cells and pulmonary artery endothelial cells was completely abolished by using cytochalasin B. Rearrangement of the actin cytoskeleton and directed migration of endothelial cells are essential in endothelial wound repair.²⁴ We found that activation of EP4 receptors by the EP4 selective agonist and PGE₂ promoted recovery of electrically wounded HMVEC-L monolayers, effects that were comparable with those of vascular endothelial growth factor. Other EP receptor agonists, such as EP2 and

EP1/3 receptor agonists, did not mimic the effect of PGE₂, whereas antagonizing the EP4 receptor completely abolished the accelerated wound healing seen with PGE₂ treatment. Similar observations were made when the endothelial barrier was compromised by pharmacologic means. Thrombin exposure of endothelial cells led to rapid stress-fiber formation across the cells and development of interendothelial gaps,⁸ which were prevented by PGE₂ or the EP4 agonist. Thus EP4 activation enhances basal barrier function but is also able to ameliorate pulmonary endothelial dysfunction induced by edemagenic stimuli.

The second major finding of the present study is reduced endothelial trafficking of neutrophils in response to EP4 engagement. Notably, PGE₂ was shown to have a protective effect in a murine model of LPS-induced lung inflammation, reducing TNF- α production and neutrophil recruitment.²⁵ Therefore we focused on the role of EP4 receptors in regulating the interaction of neutrophils with pulmonary microvascular endothelial cells. Our present data demonstrate that EP4 receptor activation decreases the adhesion of neutrophils to TNF- α -stimulated endothelial cells and reduces transendothelial migration of neutrophils. We have previously shown that neutrophils express only low levels of EP4 receptors but do not respond to PGE₂ up to concentrations of 300 nmol/L.^{26,27} However, the EP4 receptor is expressed and is highly responsive in human eosinophils, providing a direct and pronounced signal to attenuate eosinophil locomotion and activation.²⁶ The endothelial barrier-enhancing effect of EP4 stimulation most probably also applies to eosinophils and explains the high efficacy of PGE₂ at inhibiting eosinophil interaction with endothelial cells in a previous study.¹⁹ Additionally, we found that EP4 agonist and PGE₂ significantly diminished the E-selectin expression of TNF- α -activated endothelial cells. E-selectin is crucial in the initial steps of leukocyte adhesion to the endothelium,²² and decrease of E-selectin expression by EP4 receptor activation resulted in reduced adhesion of neutrophils. Therefore it appears that combined EP4-mediated endothelial barrier enhancement and reduced E-selectin expression after EP4 stimulation leads to decreased transendothelial trafficking of leukocytes.

EP4 is known to couple to G_{as} proteins, leading to stimulation of adenylyl cyclase and an increase in intracellular cAMP levels.²⁸⁻³⁰ However, recent data revealed multiple pathways of

EP4 signaling without using the classical cAMP/PKA signaling machinery.^{26,31-33} Our present work confirms that EP4 receptor activation stimulates cAMP biosynthesis in endothelial cells; however, blocking the cAMP/PKA pathway did not prevent the EP4 agonist-induced endothelial barrier enhancement or the reduction in E-selectin expression of TNF- α -stimulated endothelial cells. In contrast, our experiments suggested that the PI3K and PKC pathways are, at least partially, involved in the inhibition by PGE₂/EP4 receptors of endothelial E-selectin expression.

In conclusion, EP4 receptor is a strong inducer of pulmonary microvascular endothelial barrier function, an effect that is linked to cortical actin formation and strengthened endothelial junctions. EP4 receptor promotes endothelial wound healing and down-regulates neutrophil trafficking across pulmonary microvascular endothelial cells, although without involving cAMP/PKA signaling. All these findings suggest a great potential for EP4-selective agonists in the treatment of vascular barrier dysfunction and inflammation in patients with exacerbated pulmonary diseases.

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Clinical implications: The potential of EP4 receptors to promote the endothelial barrier might provide a novel therapeutic approach to inflammatory conditions, including allergy.

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METHODS

Reagents

Laboratory reagents were purchased from Sigma-Aldrich (Vienna, Austria), unless otherwise specified. Human IL-8 and TNF- α were from PeproTech (London, United Kingdom). PGE₂, the EP4 agonist ONO AE1-329 (Cay10598), the EP2 agonist butaprost, the EP1/3 agonist sulprostone, the extracellular signal-regulated kinase 1/2 inhibitor PD184161, the PKA inhibitor H-89, the PI3K inhibitor LY294002, and polyclonal rabbit anti-EP4 antibody were purchased from Cayman Chemicals (Ann Arbor, Mich). The EP4 antagonist ONO AE3-208 was a kind gift from ONO Pharmaceutical. Anti-rabbit and anti-mouse IgG secondary antibody conjugated with Alexa Fluor 488 and Texas Red-conjugated phalloidin were from Invitrogen (Lofer, Austria). Murine anti-VE-cadherin and anti-EP4 mAbs were from Santa Cruz Biotechnology (Santa Cruz, Calif). Vectashield/4'-6-diamidino-2-phenylindole dihydrochloride was from Vector Laboratories (Burlingame, Calif). Ultra V Blocking solution was purchased from Lab Vision (Fermont, Calif), whereas antibody diluent was from Dako (Carpinteria, Calif). The adenylyl cyclase inhibitor SQ22536 was supplied by Biomol (Hamburg, Germany), and the Rac1 inhibitor NSC 23766 was from Tocris (Ellisville, Mo). Anti-E-selectin/CD62E-PE, CellFix, and FACS-Flow were from Becton Dickinson (Vienna, Austria).

cAMP enzyme immunoassay

Endothelial cells (1.5×10^4 /well) grown in 96-well plates were starved in EBM-2 incomplete medium for 2 hours, followed by incubation in the absence or presence of 0.5 mmol/L isobutylmethylxanthine for 10 minutes. Cells were then treated with agonists for the indicated periods of time. Cell lysates together with the supernatants were processed by using the cAMP Biotrak Enzymeimmunoassay (Amersham, GE Healthcare, Vienna, Austria), according to the manufacturer's instructions.^{E1}

VE-cadherin and F-actin staining

Differentially treated endothelial monolayers grown in 1% gelatin-coated Permanox chamber slides were fixed, permeabilized, blocked for the nonspecific binding sites, and incubated with the murine VE-cadherin mAb or isotype control antibody, followed by staining with Alexa Fluor 488-conjugated secondary antibody and Texas Red-conjugated phalloidin.^{E2} Mounted samples were analyzed by using epifluorescence microscopy. Mean fluorescence

intensity of VE-cadherin expression in the junctional zone and F-actin expression in the cell periphery was quantified in the micrographs by using ImageJ software. In detail, the cell-cell junctions or the periphery of the cells were selected as the region of interest, and fluorescence intensity was measured in all endothelial cells per image. Mean fluorescence intensity of vehicle-treated endothelial cells was considered 100%.

E-selectin expression

Endothelial cell (3.5×10^4 per well) monolayers grown in 48-well plates were preincubated with signaling inhibitors for 1 hour, followed by treatment with agonists for 10 minutes. The cells were then stimulated with TNF- α for 4 hours. Cells were stained with phycoerythrin-labeled E-selectin antibody for 30 minutes at 4°C in the dark. The expression of E-selectin was quantified by means of flow cytometry.^{E3}

EP4 receptor expression

Endothelial cells were fixed and permeabilized, and nonspecific binding sites were blocked. Samples were then incubated with the specific murine EP4 primary antibody, followed by staining with Alexa Fluor 488-labeled goat anti-mouse secondary antibody.^{E2} Cells were analyzed by using flow cytometry.

For Western blotting, endothelial cells grown in 6-well plates were lysed. Total cell lysates (20 μ g) were separated on 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked in 5% skimmed milk/PBS Tween 1% and incubated with primary murine anti-EP4 mAb at 4°C for 18 hours, followed by incubation with horseradish peroxidase-conjugated anti-mouse antibody for 2 hours. Signals were detected by using ECL Plus Western blotting detection reagent (Amersham, GE Healthcare).^{E2}

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- E3. El-Gamal D, Holzer M, Gauster M, Schicho R, Binder V, Konya V, et al. Cyanate is a novel inducer of endothelial icam-1 expression. *Antioxid Redox Signal* 2012; 16:129-37.

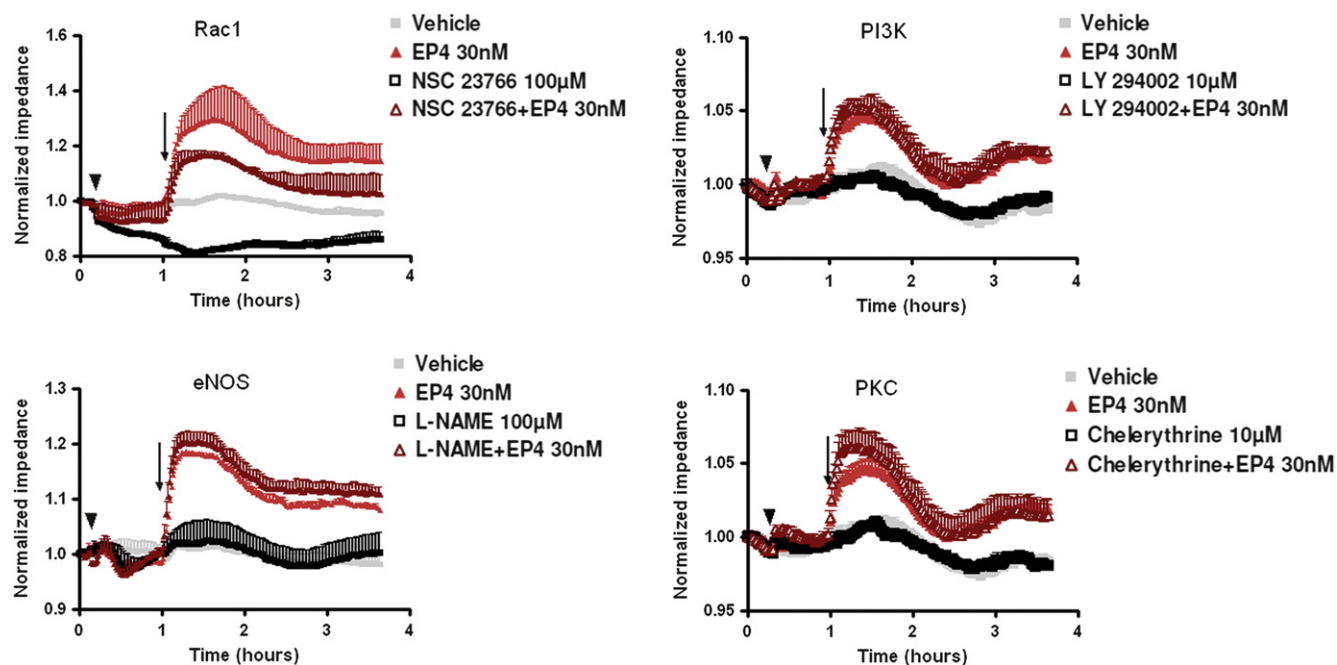


FIG E1. Inhibition of Rac1, PI3K, endothelial nitric oxide synthase (*eNOS*), and PKC pathways does not influence the EP4-induced barrier enhancement. HMVEC-Ls grown on gelatinized gold microelectrodes were serum starved for 1 hour, followed by incubation with the Rac1 inhibitor NSC 23766 (100 μmol/L), the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (10 μmol/L), the endothelial nitric oxide synthase inhibitor L-NAME (100 μmol/L), and PKC inhibitor chelerythrine (1 μmol/L) for 1 hour, as indicated by the *arrowheads*. Afterward, endothelial monolayers were treated with 30 nmol/L of the EP4 agonist ONO AE1-329 or its vehicle (*arrows*). Data show normalized impedance means + SEMs as representative of 3 independent experiments.