Prostaglandin E2-EP4 Receptor Promotes Endothelial Cell Migration via ERK Activation and Angiogenesis in Vivo*

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Prostaglandin E2 (PGE2), a major product of cyclooxygenase, exerts its functions by binding to four G protein-coupled receptors (EP1–4) and has been implicated in modulating angiogenesis. The present study examined the role of the EP4 receptor in regulating endothelial cell proliferation, migration, and tubulogenesis. Primary pulmonary microvascular endothelial cells were isolated from EP4flox/flox mice and were rendered null for the EP4 receptor with adenoCre virus. Whereas treatment with PGE2 or the EP4 selective agonists PGE1-OH and ONO-AE1–329 induced migration, tubulogenesis, ERK activation and cAMP production in control adenovirus-transduced endothelial EP4flox/flox cells, no effects were seen in adenoCre-transduced EP4flox/flox cells. The EP4 agonist-induced endothelial cell migration was inhibited by ERK, but not PKA inhibitors, defining a functional link between PGE2-induced endothelial cell migration and EP4-mediated ERK signaling. Finally, PGE2, as well as PGE1-OH and ONO-AE1–329, also promoted angiogenesis in an in vivo sponge assay providing evidence that the EP4 receptor mediates de novo vascularization in vivo.

Angiogenesis, the process of new blood vessel formation from pre-existing vessels, is a multistep event that requires endothelial cell proliferation, migration, and tube formation. Angiogenesis is controlled by diverse factors, including cytokines, growth factors, as well as cyclooxygenase-2-derived eicosanoids (1, 2). The pro-angiogenic effects of cyclooxygenase-2 are mediated primarily by three products of arachidonic acid metabolism: thromboxane A2, prostaglandin E2 (PGE2), and prostaglandin I2. These pro-angiogenic eicosanoids directly stimulate the synthesis of angiogenic factors, promote vascular sprouting, migration, tube formation, as well as enhance endothelial cell survival (1, 2).

PGE2 exerts its cellular effects by binding to four distinct E-prostanoid receptors (EP1–4) that belong to the family of seven transmembrane G protein-coupled rhodopsin-type receptors (3). Even though there is similar signaling mechanisms among these receptors, it is clear that each receptor has different and often opposing biological effects (4). For example, although the EP2 and EP4 receptors are both Gs coupled receptors and up-regulate intracellular cAMP levels, they mediate differential phosphorylation of cAMP response element-binding proteins (5). In addition, following activation, these two receptors exert different downstream effects on important intracellular mediators, including the PI3K and ERK pathways (6, 7). Moreover, the EP3 receptor usually counteracts EP2- and EP4-mediated up-regulation of cAMP by preferentially coupling to Gi proteins (3).

Some information regarding the role of PGE2 in angiogenesis has been obtained using cancer models in mice where the receptors have been deleted by homologous recombination. In this context, mice lacking the EP2 receptor produce significantly fewer and less vascularized tumors than wild type mice in a two-stage skin carcinogenesis protocol (8), and the EP2 receptor was demonstrated to directly contribute to endothelial cell migration and survival (9). Similarly, EP3-null mice exhibit decreased tumor growth and tumor-associated angiogenesis compared with wild type mice following injection of sarcoma or lung carcinoma cells (10). In contrast, the EP1 receptor does not appear to play a role in tumor-associated angiogenesis (11) and, with the exception of one in vivo study (12), there is scant information on the direct role of EP4 receptor in angiogenesis and endothelial cell function.

To characterize the contribution of the EP4 receptor in endothelial cell biology, we have undertaken studies utilizing primary pulmonary microvascular endothelial cells isolated from EP4flox/flox mice (13), which were rendered null for the EP4 receptor by in vitro treatment with adenoCre virus. The present study provides evidence that 1) primary endothelial cells express the EP4 receptor; 2) this receptor directly controls endothelial cell migration and tubulogenesis but not proliferation in vitro; 3) activation of ERK is necessary to promote the EP4-mediated endothelial cell migration; and 4) activation of the EP4 receptor by selective agonists promotes angiogenesis in vivo. Thus, the EP4 receptor not only plays a direct role in endothelial cell functions in vitro, but it also mediates angiogenesis in vivo.
EXPERIMENTAL PROCEDURES

Generation of EP4-null Endothelial Cells—Primary murine endothelial cells were isolated from EP4<sup>flox/flox</sup> mice (13) as described previously (14). Briefly, the lung vasculature was perfused with PBS, 2.5 mM EDTA followed by 0.25% trypsin, 2.5 mM EDTA via the right ventricle. Lungs were removed and incubated at 37 °C for 20 min. The visceral pleura was subsequently trimmed and the perfusion was repeated. Primary endothelial cells were recovered and grown on tissue culture plastic in EGM-2-MV containing 5% FCS (Clonetics). Cells at passages 2–4 were used for experiments.

For the generation of EP4-null endothelial cells, EP4<sup>flox/flox</sup> cells were seeded in 6-well plates (10<sup>5</sup> cells/well) and incubated with 0.5 ml serum-free medium containing 1 × 10<sup>12</sup> multiplicity of infection AdenoCre (AdCre) or β-galactosidase (β-AdGal) adenovirus. After 8 h, 1.5 ml of complete medium was added to the wells. After 3 days the cells were transduced again with AdCre or β-AdGal adenovirus as indicated above with a total of three independent treatments. This procedure led to a ∼80–90% reduction of EP4 mRNA and protein expression in AdCre-treated cells (see Fig. 2 for details).

RT- and Real-time RT-PCR—RNA was isolated from EP4<sup>flox/flox</sup> endothelial cells transduced with β-AdGal or AdCre as indicated above using TRIzol reagents. RNA samples were reverse-transcribed using a SuperScript II<sup>TM</sup> kit and oligo(dT) (12–18 bp) and 100 ng of first strand cDNA was used for real-time PCR. EP4 and 18 S primers as well as fluorescent probes were purchased from Applied Biosystems (Foster City, CA; 18 S catalogue number 4319413E and EP4 catalogue number Mm00436053_m1). The EP4 and 18 S probes were labeled at the 5′-end with the reporter fluorophores FAM and VIC respectively and at the 3′-end with a non-fluorescent quencher. Quantitative PCR was performed in a real-time format on the 7700 ABI Prism Sequence Detector (Applied Biosystems). The cycling conditions were as follows: 2 min at 50 °C followed by 40 cycles at 95 °C for 15 s (denaturation step) and 58 °C for 60 s (annealing and extension). Data were analyzed using the comparative Ct method, as described in Applied Biosystems User Bulletin and detailed by Livak and Schmittgen (15). Data were analyzed with Student’s t test, and a level of p < 0.05 was considered statistically significant.

For the RT-PCR assay, 100 ng of total RNA and utilized to amplify EP1, EP2, EP3, EP4, and β-actin fragments using Supermix PCR kit (Invitrogen). PCR conditions are as follows: 2 min at 50 °C followed by 40 cycles at 95 °C for 15 s (denaturation step) and 58 °C for 60 s (annealing and extension). Data were analyzed using the comparative Ct method, as described above using TRIzol reagents. RNA samples were reverse-transcribed using a SuperScript II<sup>TM</sup> kit and oligo(dT) (12–18 bp) and 100 ng of first strand cDNA was used for real-time PCR.

Proliferation Assay—EP4<sup>flox/flox</sup> endothelial cells, transduced with β-AdGal or AdCre as described above, were plated in complete medium onto 96-well plates (10<sup>4</sup> cells/well). After 12 h the cells were incubated with serum-free medium for further 24 h and subsequently incubated with serum free medium containing [<sup>3</sup>H]thymidine (10 μCi/ml) with or without 1 μM PGE<sub>2</sub>, PGE<sub>1</sub>-OH, Butaprost, 17-phenyl-ω-trinor-PGE<sub>2</sub> (Cayman Chemicals, Ann Arbor, MI), ONO-AE1–329 (from Dr. T. Maruyama, ONO Pharmaceuticals, Osaka, Japan), MB-28767 (Rhone-Poulenc Rorer). Twenty-four hours later, the cells were collected and the amount of incorporated [<sup>3</sup>H]thymidine was analyzed as described previously (14). Four independent experiments with quadruplicate samples were performed.

Migration Assay—Cell migration was assayed using transwell plates fitted with 8-μm membrane filters (Corning Ware). Lower wells were incubated with matrigel (5 μg/ml) at 4 °C for 12 h and then incubated at 37 °C for 1 h with 1% bovine serum albumin in PBS to inhibit nonspecific cell migration. Serum-free medium with or without PGE<sub>2</sub>, PGE<sub>1</sub>-OH, ONO-AE1–329, MB-28767, Butaprost, 17-phenyl-ω-trinor-PGE<sub>2</sub> (1 or 10 μM each), or 10% FCS was then added to the lower wells, while β-AdGal- or AdCre-treated endothelial cells (5 × 10<sup>4</sup> cells in 300 μl of serum-free medium) were added to the upper wells.

To determine the contribution of ERK, PKA, or PI3K to prostanoid-induced migration, serum-starved endothelial cells (to minimize ERK and Akt activation, as well as cAMP production) were allowed to migrate as indicated above in the presence or absence of the ERK inhibitor PD98059 (Sigma, 10 μM), the PKA inhibitor H89 (Calbiochem, 10 μM), or the PI3K inhibitor LY294002 (Calbiochem, 5 μM). After 6 h at 37 °C, cells on the top of the filter were removed by wiping, and the filters were then fixed in 4% formaldehyde in PBS. Migrating cells were stained with 1% crystal violet, and five randomly chosen fields from duplicate wells were counted at 400× magnification. Three independent experiments were performed in duplicate.

Matrigel-based Capillary Formation Assay—Capillary-like formation was analyzed as described (16). Briefly, 96-well plates were coated with 50 μl of Matrigel and incubated 30 min at 37 °C. Serum-starved β-AdGal- or AdCre-treated endothelial cells (1 × 10<sup>4</sup>) were plated on solidified Matrigel in 200 μl of serum-free medium with or without PGE<sub>2</sub>, PGE<sub>1</sub>-OH, ONO-AE1–329, MB-28767, Butaprost (1 or 10 μM each), or 10% FCS. Capillary-like structures were recorded (three images per gel per treatment) hourly for a period of 10 h and representative images taken 6 h after plating are shown. To quantify capillary-like network formation, cellular nodes were defined as junctions linking at least three cells, and they were counted from digital images. Three independent experiments were performed in duplicate with a total of 18 images analyzed per treatment.

Measurement of cAMP—EP4<sup>flox/flox</sup> endothelial cells untreated or transduced with AdCre were plated in complete medium onto 96-well plates (5 × 10<sup>4</sup> cells/well) for 24 h after which the cells were incubated in serum-free medium containing the phosphodiesterase inhibitor isobutylmethylxanthine (250 μM). After 12 h the cells were incubated in PBS containing 250 μM isobutylmethylxanthine with or without PGE<sub>2</sub>, PGE<sub>1</sub>-OH, ONO-AE1–329, MB-28767, Butaprost (1 μM each), or forskolin (10 μM, positive control). After 30 min the intracellular lev-
els of cAMP were determined via commercial enzyme-linked immunosorbent assay assays (Discoverx) and expressed in nmol/liter. Three independent experiments were performed in triplicate. Untreated EP4flox/flox cells were used for the experiment instead of /H9252-AdGal-transduced EP4flox/flox cells as this enzyme-linked immunosorbent assay kit is based on a /H9252-galactosidase-dependent assay.

**Western Blot Analysis**—To determine the expression of EP4 protein, membrane fractions were isolated from /H9252-AdGal- and AdCre-treated endothelial cells as follows. Cells were lysed in lysis buffer (15 mM HEPES, pH 7.6, 5 mM EDTA, 5 mM EGTA, and 2 mM phenylmethylsulfonyl fluoride) and passaged through a 21-gauge needle. The cell lysates were subsequently layered on a 60% sucrose cushion and centrifuged at 150,000 g for 1 hour at 4 °C. The enriched membrane fraction at the top of the sucrose cushion was collected and passed through a 26-gauge needle. Equal amount of membranes were resolved by SDS-PAGE (10% gels, 50 μg membrane/lane) and transferred to Immobilon-P membranes (Millipore). Membranes were incubated with a rabbit anti-human EP4 (C-terminal amino acids 459–488, Cayman) able to cross-react with mouse EP4 (17) and anti-N-cadherin antibody (1:1,000; Santa Cruz Biotechnology) to verify the purity and equal loading of the subcellular fractionation products. To evaluate the effects of prostanoids on ERK, p38, and Akt phosphorylation semiconfluent /β-AdGal- and AdCre-treated endothelial cells were serum-starved for 24 h and then treated with the PGE₂, PGE₂-OH, ONO-AE₁–329, MB-28767, Butaprost (1 μM or 10 μM each), or 10% FCS for 0 and 15 min. The cells were washed with PBS and lysed in 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100 and centrifuged for 10 min at 14,000 rpm. Cell lysates were resolved by SDS-PAGE (10% gels; 30 μg of total protein/lane) and transferred to Immobilon-P membranes. Membranes were incubated with a rabbit anti-phospho-ERK, anti-phospho-p38, or anti-phospho-Akt antibody (all from Cell Signaling Technology) followed by the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactive proteins were visualized using an ECL kit (Pierce). Total ERK, p38, and Akt content were verified by stripping the membranes in 50 mM Tris-HCl, pH 6.5, containing 2% SDS and 0.4% β-mercaptoethanol for 1 hour at 55 °C and re-probing with a rabbit anti-Akt antibody (Cell Signaling Technology).

**In Vivo Angiogenesis**—The subcutaneous sponge model was used to determine the effects of prostanoids on in vivo angiogenesis (16). Sterile polyvinyl acetal CF-50 round sponges (8 × 3 mm, a gift from Dr. J. M. Davidson, Vanderbilt University)
were implanted under the dorsal skin of C57 Black6 female mice (6 weeks of age, 20 g of body weight, \( n \) = 11005) treatment. The sponges were then injected every second day for 14 days with 50 l of either vehicle (corn oil) or PGE2, PGE1-OH, ONO-AE1–329, MB-28767, Butaprost, or 17-phenyl-trinor-PGE2 (10 \( \mu \)M). Ten minutes before sacrifice, mice were injected intravenously with 50 l of rhodamine-dextran (M \( r \) 65, 2% in PBS, Sigma) to label blood vessels (16), and the sponges were subsequently collected and analyzed under an epifluorescence microscope. Rhodamine-dextran-positive structures were imaged, the color images converted to black and white pictures using Photoshop (Adobe) and processed as described (16). Vascularity within sponges was expressed as a percentage of area occupied by rhodamine-dextran-positive structures per microscopic field. Three images/sponge with a total of 12 images per treatment were used for analysis.

Statistical Analysis—The Student’s t test was used for comparisons between two groups, and analysis of variance using Sigma-Stat software was used for statistical differences between multiple groups. \( p \) < 0.05 was considered statistically significant.

RESULTS

The EP4 Receptor Is Pro-angiogenic in Vivo—To test the contribution of the EP receptors to in vivo angiogenesis, we utilized a subcutaneous sponge model (16). Inert sponges, implanted subcutaneously in the back of adult mice, were injected every other day with either vehicle (oil), PGE2, the selective EP4 agonists PGE1-OH and ONO-AE1–329; the EP2 selective agonist MB-28767; the EP2 agonist Butaprost; or the EP1 agonist 17-phenyl-\( \omega \)-trinor-PGE2 (10 \( \mu \)M each). After 14 days, the sponges were isolated and vascular density determined by direct observation and analysis of vessel-associated rhodamine-dextran fluorescence. With the exception of MB-28767 and 17-phenyl-\( \omega \)-trinor-PGE2, all the prostanoids tested were able to induce in vivo angiogenesis (Fig. 1, A–C). Significantly greater vascularization was observed in sponges injected with either PGE2 or the EP4 active agonists PGE1-OH or ONO-AE1–329 compared with sponges injected with the EP2 agonist Butaprost (Fig. 1, A–C). These results indicate that although both EP2 and EP4 receptors are able to promote de novo blood vessel formation, EP4 is the most potent pro-angiogenic receptor (Fig. 1, A–C).

Primary Murine Endothelial Cells Express the EP4 Receptor, Which Promotes Cell Migration but Not Proliferation—To determine whether the in vivo EP4 agonist-induced angiogenesis was due to a direct effect of these ligands on endothelial cell function, we analyzed which EP receptors are expressed in cul-
tured endothelial cells by performing RT-PCR analysis on primary EP4flox/flox endothelial cells transduced with control β-AdGal. As shown in Fig. 2A, β-AdGal-transduced endothelial cells expressed mRNA for EP1, EP2 and EP4 subtypes, while EP3 mRNA was not detected.

As the pro-angiogenic receptor EP4 is expressed on endothelial cells, we determined its role in mediating endothelial cell proliferation, migration and tubulogenesis by comparing endothelial cells derived from EP4flox/flox mice transduced with either β-AdGal or AdCre virus. As shown in Fig. 2A, we first demonstrated that the levels of EP4 mRNA, but not EP1 or EP2 were significantly decreased in AdCre-transduced endothelial cells. Real-time PCR confirmed that the levels of EP4 mRNA in β-AdGal-treated endothelial cells were ~10-fold higher than those detected in AdCre-treated cells (Fig. 2B). Decreased expression of the EP4 receptor in AdCre-treated endothelial cells was also confirmed by Western blot analysis of membrane enriched fractions (Fig. 2C). Thus, endothelial cells express the EP4 receptor and treatment of EP4flox/flox endothelial cells with AdCre selectively down-regulates the expression of this receptor without affecting the levels of the other PGE2 binding receptors.

As activation of the EP4 receptor promotes angiogenesis in vivo (Fig. 1) and this receptor controls cell growth and survival in different cell types (7, 18), we examined whether the EP4 receptor contributes to PGE2-mediated endothelial cell proliferation. For this reason, endothelial cells were treated with PGE2, PGE1-OH, ONO-AE1–329, Butaprost, MB-28767, or 17-phenyl-ω-trinor PGE2 (1 μM each). Unlike serum-treatment, none of the specific agonists induced cell proliferation in either β-AdGal- or AdCre-transduced endothelial cells (Fig. 3A). This result suggests that PGE2-induced activation of the EP receptors is not sufficient to promote endothelial cell proliferation.

In contrast, PGE2, PGE1-OH and ONO-AE1–329 used at 1 μM significantly stimulated migration of β-AdGal-, but not AdCre-transduced endothelial cells, suggesting that low doses of PGE2 promotes endothelial cell migration via activation of the EP4 receptor (Fig. 3B). The EP3 agonist MB-28767 did not promote endothelial cell migration (Fig. 3B). Moreover, no significant differences in basal or serum-induced cell migration were observed between β-AdGal- and AdCre-treated cells (Fig. 3B). Interestingly, 17-phenyl-ω-trinor PGE2 used at both low (1 μM) and high (10 μM) doses did not promote endothelial cell migration (Fig. 3B), suggesting that, although endothelial cells express EP1 mRNA (Fig. 2A), this receptor does not play a direct role in endothelial cell migration. Finally, activation of the EP2 receptor with high (10 μM) but not low (1 μM) doses of Butaprost promoted migration of either β-AdGal- or AdCre-treated endothelial cells (Fig. 3B), confirming the data that high doses of EP2 ligands are able to promote endothelial cell migration (9). We were unable to determine whether high doses of PGE2 (i.e. 10 μM) could lead to migration in both β-AdGal- and AdCre-treated cells, as this dose was cytotoxic for endothelial cells of both genotypes.

The EP4 Receptor Promotes Capillary-like Structure Formation—The role of EP4 receptor activation in the formation of capillary-like structures was assessed by plating endothelial cells on solidified Matrigel in the absence of serum. Within 6 h, β-AdGal-treated cells formed capillary-like structures more efficiently than AdCre-treated cells at baseline, suggesting that the EP4 receptor plays a role in endothelial branching (Fig. 4, A and B). PGE2, PGE1-OH, and ONO-AE1–329 (1 μM each) sig-
nificantly increased the formation of capillary-like structures only in β-AdGal-transduced cells (Fig. 4, A and B), thus confirming that at low doses PGE₂ seems to support tubulogenesis primarily via activation of the EP4 receptor. In contrast, no significant changes were observed with MB-28767 (used as negative control). Finally, whereas Butaprost at 1 μM failed to stimulate endothelial cell tubulogenesis, at 10 μM it stimulated capillary-like structure formation in both EP4 expressing and EP4 non-expressing endothelial cells (Fig. 4, A and B). These results indicate that although both EP2 and EP4 receptors can mediate capillary-like structure formation, engagement of EP2 receptor requires high doses of ligand.

Activation of the EP4 Receptor Leads to Increased Intracellular cAMP Levels and ERK Activation—Activation of either EP2 and EP4 receptors, which are both expressed in primary endothelial cells (Fig. 2A), stimulates the production of intracellular cAMP in a cell specific manner (3). Moreover, PGE₂ promotes angiogenesis by increasing levels of cAMP in human endothelial cells (19), although the receptor that mediates these effects is unknown. For these reasons, we analyzed whether the EP2 and EP4 receptors can stimulate intracellular cAMP levels in microvascular endothelial cells and which of these two receptors exerts this function. As shown in Fig. 5A, treatment with PGE₂ and the EP2 agonist Butaprost (both at 1 μM) increased intracellular levels of cAMP in both Ep4flox/flox and AdCre-transduced Ep4flox/flox endothelial cells. In contrast, treatment with the EP3 agonist MB-28767 (negative control) failed to stimulate cAMP production in either cell type. Conversely, treatment with the EP4 agonists PGE₁-OH or ONO-AE1–329 stimulated cAMP production only in EP4flox/flox endothelial cells. Thus, this finding suggests that activation of EP2 and EP4 receptors by low doses of ligands induce cAMP production in endothelial cells.

Based on observations that stimulation of the EP4 receptor leads to phosphorylation of ERK and Akt (6, 7) and PGE₂ promotes p38 MAPK activation (20), we determined the contribution of the EP4 receptor to the activation of these protein kinases in primary endothelial cells. Incubation of β-AdGal- and AdCre-transduced EP4flox/flox cells with PGE₂, PGE₁-OH, or ONO-AE1–329 (1 μM each) resulted in a marked increase in ERK phosphorylation, and to a lesser extent Akt and p38 MAPK activation, only in endothelial cells expressing the EP4 receptor (Fig. 5B). In contrast, treatment with 1 μM MB-28767 (negative control) or 1 μM Butaprost did not promote phosphorylation of these three kinases (Fig. 5B) in either β-AdGal- or AdCre-transduced cells. Interestingly, when used at 10 μM Butaprost stimulated ERK activation in both cell types (Fig. 5B), thus paralleling the finding that high doses of EP2 ligand can promote both migration (Fig. 3B) and capillary-like structure formation (Fig. 4).

FIGURE 4. The EP4 receptor contributes to capillary like structure formation. A, serum-starved β-AdGal- and AdCre-transduced EP4flox/flox endothelial cells were plated onto Matrigel in the absence (vehicle) or presence of PGE₂, PGE₁-OH (E₁-OH), ONO-A1–329 (ONO), MB-28767 (MB), Butaprost (Buta) (1 or 10 μM each), or 10% FCS. Representative images of capillary-like structures taken 6 h after plating are shown. B, capillary network formation was quantified as described under “Experimental Procedures.” Values are the mean ± S.D. calculated for 18 images per treatment. * and ** are the same as described in the legend to Fig. 3.
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FIGURE 5. PGE2 induces intracellular cAMP levels and ERK activation in endothelial cells. A, levels of intracellular cAMP 30 min after the stimulation of EP4flox/flox and AdCre-transduced EP4flox/flox endothelial cells with PGE2, PGE1-OH (E1-OH), ONO-A1–329 (ONO), MB-28767 (MB), and Butaprost (Buta) (1 μM each) were determined as described under “Experimental Procedures.” The values represent the mean ± S.D. of one representative experiment performed in triplicate and are expressed as fold changes relative to vehicle-untreated cells. * and ** are as in Fig. 3. B, serum-starved β-AdGal (“β”) and AdCre (“A”) transduced EP4flox/flox endothelial cells were either kept untreated (vehicle) or treated for 15 min with PGE2, PGE1-OH (E1-OH), ONO-A1–329 (ONO), MB-28767 (MB), Butaprost (Buta) (1 or 10 μM each), or 10% FCS. Levels of phosphorylated ERK, Akt, and p38 MAPK were determined in total cell lysates (30 μg/lane) by Western blot analysis. Membranes were stripped and re-probed with anti-Akt antibodies to verify equal loading.

Thus, this data indicates that activation of EP4 receptor by low doses of ligand results in both cAMP production and ERK activation. In contrast, activation of EP2 by low doses of ligand only stimulates cAMP production, while high doses of ligand are required for EP2-mediated ERK activation.

Activation of ERK Is Necessary to Support EP4-mediated Endothelial Cell Migration—To determine whether activation of ERK and/or PKA was necessary to support EP4-mediated endothelial cell migration, serum-starved β-AdGal- and AdCre-transduced EP4flox/flox endothelial cells (to minimize ERK activation and cAMP production) were allowed to migrate toward 1 μM PGE2 or ONO-AE1–329 (both able to stimulate cAMP production and ERK activation, see Fig. 5) or 1 μM Butaprost (only able to promote cAMP production but not ERK activation, see Fig. 5) in the presence or absence of PD98059 (a MEK1/ERK inhibitor), H89 (a PKA inhibitor), or LY294002 (a PI3K inhibitor, used as negative control). Neither PGE2 or ONO-AE1–329 were able to stimulate β-AdGal-transduced endothelial cell migration following ERK pathway inhibition (Fig. 6). In contrast, both prostanoids promoted migration of β-AdGal-treated cells cultured in the presence of the PKA inhibitor H89 or the PI3K inhibitor LY294002, suggesting ERK, but not PKA or PI3K is involved the EP4-mediated cell migration. Furthermore, 1 μM Butaprost failed to promote cell migration of β-AdGal-transduced endothelial cells with or without treatment with the ERK, PKA, or PI3K inhibitors (Fig. 6) further supporting a role for ERK, rather than the PKA pathway in endothelial cell migration. As expected, the basal migration of AdCre-transduced endothelial cells did not change following treatment with 1 μM prostanoids alone or in combination with the kinase inhibitors (Fig. 6). Interestingly, when used at 10 μM, Butaprost stimulated migration of both β-AdGal-treated and AdCre-treated cells, and this effect was prevented only by the ERK inhibitor (Fig. 6), further supporting (i) a role for ERK, rather than the PKA or PI3K pathway in endothelial cell migration; (ii) that engagement of EP2 receptor by high doses of ligand can promote endothelial cell migration; and (iii) both EP2 and EP4 receptors require ERK activation to promote endothelial cell migration.

DISCUSSION

PGE2 has been shown to mediate diverse cell functions via four distinct transmembrane receptors, designated EP1, EP2, EP3, and EP4. In this study we provide evidence for a role of the EP4 receptor in endothelial cell function by comparing the response of β-AdGal- versus AdCre-transduced primary EP4flox/flox endothelial cells to PGE2 or EP4-selective agonists. We provide evidence that (1) primary endothelial cells express the EP4 receptor; (2) this receptor does not stimulate endothelial cell proliferation but rather promotes endothelial cell migration and tubulogenesis; (3) although activation of the EP4 receptor by selective agonists significantly increases both ERK phosphorylation and intracellular cAMP levels; only ERK activation promotes the EP4-mediated endothelial cell migration; and (4) EP4 agonists are highly angiogenic in vivo. Thus the EP4 receptor plays a direct role in mediating endothelial cell functions in vitro and, most importantly, promotes angiogenesis in vivo.

The role of PGE2 in endothelial cell proliferation is controversial. Our data demonstrate that mouse microvascular endothelial cell proliferation is not stimulated by PGE2 and/or EP-selective agonists. Similarly, endothelial cells deficient in EP4 receptor (present study) or lacking the EP2 receptor (9) show similar basal proliferation to wild type cells and their growth is not affected by prostanoids. These observations contrast previous findings that exogenous PGE2 inhibits corneal and dermal microvascular endothelial cell growth as well as HUVEC proliferation (21, 22). Furthermore, it has been shown that low doses of PGE2 promote HUVEC proliferation in vitro by stimulating nitric oxide production (19). Thus, the effects of PGE2 on endothelial cell proliferation appear to depend on the nature of the endothelial cells themselves.

In this study we show that both EP2 and EP4 receptors control endothelial cell migration/tubulogenesis. However, low doses of EP4 agonists (i.e. 1 μM) are sufficient to promote endothelial cell migration in an EP4-specific manner, while high doses of Butaprost (i.e. 10 μM) are required to activate EP2-mediated endothelial cell migration. Moreover, the observation
that at 1 μM PGE₂ promotes endothelial cell migration via engagement of the EP4 receptor suggests that EP4 is the most pro-angiogenic among the PGE₂ receptors. The observation that high doses of Butaprost are required for EP2-mediated endothelial cell migration parallels the finding that the same dose of ligand is required to promote endothelial cell migration in wild type but not EP2-null endothelial cells (9). However, our finding that 1 μM PGE₂ promotes migration in wild type, but not EP4-null cells, contrasts with the finding that EP2-null cells fail to migrate when stimulated by 1 μM PGE₂ (9). Interestingly, the expression of the EP receptors, namely EP1, EP3 and EP4, was not investigated in EP2-null endothelial cells. Although unlikely, it is possible that in endothelial cells lacking the EP2 receptor there is down-regulation of other pro-angiogenic EP receptor, including the EP4 receptor, which might contribute to the decreased response of these cells to low doses PGE₂ stimulation. We were unable to determine whether high doses of PGE₂ (i.e. 10 μM) can promote migration in an EP2 and EP4 dependent fashion, as this dose was cytotoxic for endothelial cells.

We demonstrate that activation of EP4 receptor by ONO-AE1–329 promotes endothelial cell migration at 1 μM, 10-fold lower than the dose of EP4 agonist needed to support HUVEC migration (12). We also show that activation of the EP4 receptor in endothelial cells promotes ERK activation and cAMP production. As these two pathways are not activated in cells where the EP4 receptor is down-regulated by adenoCre treatment, our result strongly suggests that they are EP4-mediated. Furthermore, we found that one of these two signaling pathways only the ERK signaling is necessary to support EP4-mediated endothelial cell migration.

Endothelial cell migration following activation of the EP2 receptor at high doses of Butaprost could be inhibited by PD98059, suggesting that both EP2 and EP4 receptors can promote endothelial cell migration in an ERK-dependent fashion. The finding that 1 μM Butaprost failed to stimulate ERK activation in either AdCre-treated or β-AdGal-treated endothelial cells parallels the finding that in vascular smooth muscle cells only treatment with low dose EP4 agonists, but not Butaprost, caused a time-dependent ERK activation (23). In contrast, activation of EP2 by Butaprost has been shown to promote both PKA and ERK activation in human endometriotic stromal cells (24). Thus the ability of the EP2 receptor to promote ERK activation is dependent on the dose of the ligand(s) as well as the cell type.

Activation of the EP4 receptor has been shown to engage several intracellular pathways, including the ERK, PI3K, and the p38 MAPK pathways (6, 7, 25). Moreover, it has been shown that PGE₂-mediated activation of the EP4 receptor leads to ERK activation in a PI3K-dependent manner (6). In this context, we previously showed that engagement of the EP4 receptor in mouse colon carcinoma cells stimulates both ERK and Akt activation, and inhibition of either one of these pathways significantly prevents EP4-mediated tumor cell proliferation (7). However in the endothelial cells used in these studies neither PGE₂ nor the EP4 agonists PGE₁-OH and ONO-AE1–329 significantly stimulated Akt phosphorylation, suggesting that ERK is activated in a PI3K independent manner in these cells. Interestingly, it has been shown that the EP4 receptor can primarily activate the ERK signaling pathway either directly (26) or by promoting EGF receptor trans-activation (27, 28), thus not requiring the involvement of the PI3K/Akt pathway. Finally, it has been suggested that ERK can be activated by cAMP-mediated PKA activation in pancreatic (29) and uveal melanoma cells (30). However, the data provided in this study indicates that, in the endothelial cells, EP4-mediated cell migration is cAMP/PKA independent, as the PKA inhibitor H89 failed to inhibit the EP4-mediated migration.

Recent studies have shown ERK, together with the other two members of the MAPK family, namely Jun N terminus kinase

FIGURE 6. EP4 promotes endothelial cell migration via ERK activation. Serum-starved β-AdGal- and AdCre-transduced EP4flox/flox endothelial cells were plated in serum-free medium in transwells coated with 5 μg/mL Matrigel and allowed to migrate for 6 h at 37°C toward serum-free medium in the absence (vehicle) or presence of PGE₂, ONO-A1–329 (ONO), or Butaprost (Buta) (1 or 10 μM each) with or without the ERK inhibitor PD98059 (10 μM), the PKA inhibitor H89 (10 μM), or the PI3K inhibitor LY294002 (5 μM). Values are the mean ± S.D. of one representative experiment performed in triplicate. Differences between vehicle-treated and prostanoid-treated cells (*) or prostanoid-treated and prostanoid + inhibitor-treated cells (**) were significant with p < 0.05.
and p38, play a crucial role in regulating cell migration (reviewed in Ref. 31). The observation that the ERK pathway inhibitors (i.e., PD98059 and U0126) prevent the migration of cells in response to matrix proteins (32), growth factors (33), as well as prostanoids (present study) clearly define a role for ERK in governing cell movement. Although ERK can activate many downstream effectors able to control cell migration (reviewed in Ref. 31), the three most likely substrates to be activated in ERK-mediated cell migration are the myosin light chain kinase (34), calpain (35), and FAK (36). In this context, ERK can control cell movement by 1) regulating membrane protrusions and focal adhesion turnover via myosin light chain kinase phosphorylation (37), 2) promoting focal adhesion disassembly via calpain phosphorylation (35), and 3) controlling focal adhesion dynamics by regulating FAK and paxillin phosphorylation/interaction (36).

Only very low levels of p38 activation were observed in endothelial cells upon PGE2 or EP4 agonist stimulation, indicating that this MAPK family member plays little role in the EP4-mediated endothelial cell functions. This result contrasts with other findings that the EP4 receptor can activate the p38 MAPK pathway in synovial cells (25). However, this suggested involvement of EP4 in PGE2-mediated p38 MAPK activation (25) was only indirectly proven as it was based on the fact that p38 MAPK activation in synovial cells could not be inhibited by EP1, EP2, or EP3 antagonists, suggesting that an EP4-like receptor might be involved in the activation of this kinase (25). It is unclear whether synovial cells express the EP4 receptor and whether this receptor is indeed directly involved in p38 MAPK activation.

Finally, we have demonstrated that PGE2, as well as the EP4 selective agonists PGE1-OH and ONO-AE1–329 are highly angiogenic in vivo. These findings parallel the observation that ONO-AE1–329 promotes angiogenesis in a cornea assay model (12). Although both studies suggest a role for the EP4 receptor in de novo vascularization, and we provide evidence that the EP4 receptor plays a direct role in endothelial cell functions in vitro, the two in vivo studies do not allow one to determine whether the EP4-mediated vascularization is due to a direct and/or indirect effect of the agonists on endothelial cells. The insertion of sponges and/or pellets in vivo is often accompanied by an inflammatory response with macrophage infiltration. Treatment with EP4 agonists can stimulate macrophage matrix metalloproteinase-9 expression (38), which in turn could contribute to matrix degradation and de novo vascularization. Moreover, it has been shown that PGE2 can promote VEGF production in synovial fibroblasts via an EP4-mediated mechanism (39). Therefore it is possible that the EP4 agonists might stimulate the production of pro-angiogenic factors in vivo by infiltrating fibroblasts, thus only indirectly contributing to angiogenesis. To determine the direct contribution of the EP4 receptor on the de novo vascularization, mice lacking the EP4 receptor specifically in endothelial cells are needed. For this reason we have started to cross the EP4<sup>fl</sup><sub>ox</sub>/<sup>fl</sup><sub>ox</sub> mice with the Tie2-Cre mice (40), but at present we are unable to obtain viable specific endothelial cell EP4-null mice. As the total EP4-null mice die at birth due to patent ductus arteriosus (41), it might be that lack of the EP4 receptor in endothelial cells might recapitulate the phenotype of the total null mouse.

In conclusion, utilizing pulmonary endothelial cells lacking the EP4 receptor expression, we demonstrate that the EP4 receptor plays a critical role in PGE2-dependent in vitro migration/tubulogenesis which is mediated by activation of the ERK pathway. Furthermore, we show in vivo that EP4 agonists induce increased vascularization.

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