Prostaglandin E₂ Primes the Angiogenic Switch via a Synergic Interaction With the Fibroblast Growth Factor-2 Pathway

Federica Finetti,* Sandra Donnini,* Antonio Giachetti, Lucia Morbidelli, Marina Ziche

Rationale: Prostaglandin (PG)E₂ exerts temporally distinct actions on blood vessels, immediate vasodilatation, and long-term activation of angiogenesis.

Objective: To study the mechanism of PGE₂ induction of angiogenesis, we characterized its effect on fibroblast growth factor (FGF)-2 signaling in cultured endothelial cells and in ex vivo and in vivo assays of blood vessel formation.

Methods and Results: Using Western blotting assay, we demonstrated that PGE₂ induced upregulation of components of the FGF-2 pathway: FGF-2 protein, phosphorylation of FGF receptor type 1 (FGFR1), activation of FRS2α (FGFR substrate 2α), phospholipase Cγ, endothelial nitric oxide synthase, extracellular signal-regulated kinase 1/2, and the transcription factor STAT-3. Synergism between PGE₂ and FGF-2 promoted endothelial cell proliferation and robust angiogenesis in vivo, in rabbit cornea and Matrigel assays. The magnitude of the angiogenic response to PGE₂ was directly related to FGF-2 availability which determined the extent of FGFR1 activation. In fact, PGE₂ induction of angiogenesis in vitro was impaired in FGF-2/ fibroblast growth factor-2/ fibroblast growth factor receptor type1/ endothelial cells and FGFR1 blockade abrogated PGE₂ action on the endothelium, preventing the activation of FGF-2 signaling.

Conclusion: We propose a model for the angiogenic switch based on the autocrine/paracrine FGF-2/FGFR1 activation by PGE₂ and FGF-2 synergistic interaction. The synergism between the PGE₂ and FGF-2 signaling pathways here described may explain the mechanism of action of drug combinations, the most notable being cyclooxygenase inhibitors with growth factors or growth factor receptor inhibitors. (Circ Res. 2009;105:657-666.)

Key Words: prostaglandin E₂ \textbullet\ angiogenesis \textbullet\ fibroblast growth factor-2 \textbullet\ fibroblast growth factor receptor type1 \textbullet\ inflammation

Prostaglandin (PG)E₂, originally discovered along with other prostanoids for its action on blood vessels,¹ is widely recognized as a mediator of inflammation, capable of recruiting proinflammatory cells and causing pain. In the last decade, PGE₂ has acquired the profile of promoter of tumorigenesis because of its causal association with tumor growth.² Lately, PGE₂ and its synthesizing enzyme cyclooxygenase (COX)-2 have been implicated in the control of tumor invasion, a process they accomplish in cooperation with selected gene products present in primary tumors.³ The PGE₂ protumorigenic action has been attributed to its ability to activate the angiogenic switch, a process which leads to neovessel formation in tumors and which has been documented to be crucial for their continuous growth and propensity to metastasize.⁴ The angiogenic switch, a term coined by Folkman and Hanahan, defines an event finalized to activate the vascular endothelium and to produce sprouting from preexisting vessels.⁵ Although, the angiogenic switch concept has advanced our understanding of tumor biology, its operational mechanism remains poorly delineated in terms of molecules, intracellular signaling and controls involved.

As part of the renewed interest on the vascular actions exerted by PGE₂, we have recently shown that the interaction of the prostanoid with its EP3 receptor subtype, induces the angiogenic phenotype by activating the fibroblast growth factor receptor type 1 (FGFR1) pathway in microvascular endothelial cells.⁶ Several layers of complexity characterize the underlying mechanism of this activation. It involves, first, the EP3-mediated intracellular stimulation of the tyrosine kinase cSrc, which produces shedding of extracellular matrix–bound fibroblast growth factor (FGF)-2 via activation of metallo-proteases, and finally the interaction of the free ligand, FGF-2, with its receptor, FGFR1. Thus, the basis for PGE₂-induced angiogenesis lies on the excitation of the FGF-2/FGFR1 pathway, primarily designed to maintain viability of the endothelium.

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To pursue the indications provided by this work, we examined whether the interaction with the FGF-2/FGFR1 pathway might be the mechanism of the PGE2-induced angiogenic switch and the determinants of this interaction, ie, the concentrations of molecules critical for triggering the angiogenic phenotype, and the signals transducing the stimuli from cell membrane to the nucleus which are involved in neovessel formation. To mimic vasculature persistently activated by growth factors and cytokines, we have used endothelial cells wild type and transfected with fgf-2 gene to deliver FGF-2 in vitro and in vivo, and directly activated in an autocrine/paracrine manner by the growth factor itself. In these cells we measured the FGF-2-induced angiogenic potential when challenged with PGE2. We found a synergistic interaction between PGE2 and the autocrine/paracrine FGF-2/FGFR1 cycle, and by using FGF-2 expression and PGE2-transfected endothelial cells we confirmed the central role of FGF-2 overexpression in PGE2-induced angiogenesis. Moreover, we strengthened the autocrine control of the angiogenic switch by the finding that calcium-dependent endothelial nitric oxide synthase (eNOS) controls FGF-2 upregulation.

Together the results provide a mechanistic model of the PGE2-mediated angiogenic switch in FGF-2 elicited angiogenesis.

**Methods**

**Cell Lines**

Postcapillary venular endothelial cells (CVECs) were cultured as described. Mouse aortic endothelial cells (MAECs) and MAECs stably transfected with human FGF-2 cDNA (pZIPbFGF) were provided by M. Presta (University of Brescia, Italy), and cultured as reported. FGF-2 expression and PGE2-transfected endothelial cells were provided by P. Mignatti (New York University School of Medicine), and cultured as described. eNOS-null endothelial cells were provided by Prof. I. Fleming (University of Frankfurt, Germany) and cultured as described.

**Proliferation Assays**

Proliferation and cell count were performed as described.

**Western Blotting**

Western blotting was carried out as described. For details, see the expanded Methods section in the Online Data Supplement, available at http://circres.ahajournals.org.

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**Non-standard Abbreviations and Acronyms**

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADU</td>
<td>arbitrary densitometric unit</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>CVEC</td>
<td>coronary venular endothelial cell</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>EP3</td>
<td>prostaglandin E receptor 3</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>FGFR1</td>
<td>fibroblast growth factor receptor type 1</td>
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<tr>
<td>FRS2α</td>
<td>fibroblast growth factor receptor substrate 2α</td>
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<tr>
<td>L-NMMA</td>
<td>L-arginine monocarboxylate</td>
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<td>MAEC</td>
<td>mouse aortic endothelial cell</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase</td>
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<td>PGE2</td>
<td>prostaglandin E2</td>
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<tr>
<td>PLCγ</td>
<td>phospholipase Cγ</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<td>STAT-3</td>
<td>signal transducer and activator of transcription 3</td>
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**Figure 1.** PGE2 synergizes with FGF-2 in promoting endothelial cell proliferation and angiogenesis. A, inset, Western blotting for FGF-2 expression in basal condition in MAECs, pZIPbFGF, FGF2 and CVECs. The gels shown in this and following figure are representative of three obtained with similar results. A, Sparse, synchronized endothelial cells were exposed to PGE2 (1 to 1000 nmol/L). Data are reported as percentage increases over basal growth rate (n=3). *P<0.05, **P<0.01 vs control (for pZIPbFGF); #P<0.05 or ##P<0.01 vs 0.1% serum alone (for CVECs, MAECs, and FGF-2-expressing endothelial cells). B, Isobologram analysis (EC50) of CVECs treated with PGE2 (0.001 to 100 nmol/L) and/or FGF-2 (0.03 to 30 ng/mL) for 48 hours (n=3). C, Angiogenic activity of PGE2 and FGF-2 in the rabbit cornea model. Representative images of neovascularization induced by 100 ng/pellet FGF-2 (1), 500 ng/pellet PGE2 (2), or FGF-2/PGE2 in combination (3) are reported at day 10 after implant.
Immunoprecipitation
FGFRs immunoprecipitation and Western blotting analysis was performed as described. For details, see the Online Data Supplement.

Small Interfering RNA Transfection
21-nucleotide RNAs were synthesized by Qiagen (Italy). The small interfering (si)RNA sequence targeting bovine eNOS corresponding to region 3520 to 3542 relative to the start codon, and the one targeting the bovine FGF-2 corresponding to region 848 to 866 relative to the start codon were used. Transient transfection of siRNA was carried out using Lipofectamine (Invitrogen) as described. For details on sequences and experimental procedure, see the Online Data Supplement.

Immunofluorescence Analysis
CVECs (2.5×10⁴ cells/well on glass cover-slips placed into 24 multiwell plates) were incubated in 0.1% serum with PGE2 (100 nmol/L), or FGF-2 (20 ng/mL). After 15 minutes cells were fixed and incubated with monoclonal antibodies against phospho–STAT-3 (signal transducer and activator of transcription 3) Ser(727), or –phospho–tyrosine antibodies (Cell Signaling); anti–FGF-2, vascular endothelial growth factor, epidermal growth factor (Peprotech); anti–phospho–STAT-3, –FRS2α, –phospholipase (PL)Cγ, –eNOS(Ser1177), and –phospho-tyrosine antibodies (Cell Signaling); anti–FGF-2 neutralizing, anti–FGFRI, –II, –III, –IV, and –FGF-2 antibodies (Upstate). Further details are available in the Online Data Supplement.

Endothelial Tube Formation From Aortic Rings
Pseudocapillary sprouting from mouse aorta (1- to 2-mm) rings was evaluated as described. For details, see the Online Data Supplement.

Angiogenic Assays
Angiogenesis was studied in the cornea of New Zealand rabbits (Charles River) and in the Matrigel plug assay as described. For details, see the Online Data Supplement.

Reagents
Reagents were obtained from Sigma, except: U0126, l-Nω-monomethyl arginine (L-NMMA), SU5402, PyYLKTK-mts, and U0126 (Calbiochem-Novabiochem); FGF-2, vascular endothelial growth factor, epidermal growth factor (Peprotech); anti–phospho–STAT-3, anti–STAT-3, –FRS2α, –phospholipase (PL)Cγ, –eNOS(Ser1177), and –phospho-tyrosine antibodies (Cell Signaling); anti–FGF-2 neutralizing, anti–FGFRI, –II, –III, –IV, and –FGF-2 antibodies (Upstate). Details on sequences and experimental procedure, see the Online Data Supplement.

Statistics
Statistical analysis was performed using Student’s t test for unpaired data or by analysis of variance (ANOVA); P<0.05 was considered statistically significant. The synergism between PGE2 and FGF-2 was evaluated by isobologram analysis.

Results
PGE2 and FGF-2 Synergize to Promote Angiogenesis
In a recent report, we have described the proangiogenic action of PGE2, illustrating its activation of the FGFR1 through FGF-2 mobilization. Here, we investigated whether the PGE2 proliferative action might depend on the availability of endogenous FGF-2. To investigate this, we used endothelial cells characterized by different intracellular FGF-2 content: CVECs, MAECs, and pZIPbFGF; an engineered counterpart of MAECs overexpressing FGF-2 (mainly the 18-kDa isoform), or FGF-2−/− endothelial cells. Basal expression of FGF-2 in these cells is reported at the top of Figure 1A. Incubation of CVECs, MAECs, or pZIPbFGF with PGE2, at concentrations spanning from subnanomolar to micromolar, elicited a concentration dependent proliferation (Figure 1A). FGF-2 expression levels did not change with passages in culture (data not shown). In pZIPbFGF cells, growth was detectable at prostanoid concentrations as low as 1 nmol/L (counted cells: control: 132±3.6; PGE2: 135±6 [0.1 nmol/L]; 166±20 [1 nmol/L]; 190±22 [10 nmol/L], and 237±20 [100 nmol/L]). In contrast, MAECs and CVECs began to significantly proliferate at 10 nmol/L of PGE2 (counted cells: 73±5 versus 50±18 as control for MAECs, and 146±5 versus 98±6 as control for CVECs), reaching maximal growth at 100 nmol/L (counted cells: 96±5 and 189±9, respectively, for MAECs and CVECs). In FGF-2−/− cells, we did not detect any proliferative effect by PGE2 at all concentrations tested (Figure 1A). Differences in sensitivity among cell lines to PGE2 imply that the FGF-2 availability is essential for its proliferative effect, and it critically determines the magnitude of the PGE2 response.

In light of the above results, we wondered whether a synergic interaction between PGE2 and FGF-2 was operant in endothelial cells. To examine this possibility we conducted cell proliferation experiments in CVECs by using a wide range of PGE2 concentrations (0.001 to 100 nmol/L) in the presence of mounting FGF-2 concentrations (0.03 to 30 ng/mL). Interestingly, in this cellular model, endogenous production of PGE2 was undetectable in absence of admin-
istration of arachidonic acid (data not shown), indicating that the exogenous prostanoid is the only species involved in the synergism. Data examined by the isobologram analysis provided clear evidence of synergic interaction between PGE2 and FGF-2 functional responses (Figure 1B). The PGE2/FGF-2 synergism was specific because other growth factors, such as vascular endothelial growth factor or epidermal growth factor used at noncompetent concentrations of 0.5 ng/mL failed to synergize with PGE2 (1 nmol/L) (Online Table I). This synergism was also observed in vivo in the rabbit avascular cornea, in which simultaneous delivery of PGE2 (500 ng) and FGF-2 (100 ng) produced a flourishing capillary network (Figure 1C) and a rise of the angiogenic score (12 versus 6 and 5.4 at 10 days for the combination versus PGE2 and FGF-2 alone, respectively), whereas each single compound elicited a weaker stimulation (Figure 1C).

PGE2/FGF-2 Signaling Translates in Endogenous FGF-2 Upregulation

In light of the synergism seen above, we wondered whether the interaction would apply to the control of endothelial FGF-2 production, because upregulation of the growth factor is often associated with activation of the angiogenic phenotype. To examine this hypothesis, we measured FGF-2 protein expression in endothelial cells exposed to increasing PGE2 concentrations. In CVECs, the prostanoid elicited a concentration and time-dependent FGF-2 overexpression, maximal activity being at 100 nmol/L. In these cells, the addition to the medium of FGF-2 (0.3 ng/mL) enhanced the endogenous growth factor production, maximal expression being reached at 1 nmol/L PGE2, indicating that the prostanoid synergizes with FGF-2 to trigger FGF-2 synthesis (Figure 2). The relevance of FGF-2 for the expression of the angiogenic phenotype was seen in functional experiments measuring cell proliferation. In fact, quenching the FGF-2 action, either through a neutralizing antibody anti–FGF-2 (0.6 μg/mL) or siRNA interference technique, abolished the PGE2 proliferative response (Figure 3A, top). Additional proof that FGF-2 is required for the PGE2 proangiogenic activity was obtained by using FGF-2–null (FGF-2/−/−) endothelial cells. In these cells, application of PGE2 up to 100 nmol/L failed to promote proliferation, whereas addition to the medium of FGF-2 (0.3 ng/mL) together with PGE2 (1 to 100 nmol/L) restored the prostanoid proliferative ability (Figure 3B).

In Vivo Synergism Between PGE2 and FGF-2

Reportedly, the vasculature is activated by growth factors and cytokines, because endothelial cells inherently produce abundant growth factors. Here, we investigated the in vivo relevance of the activation of FGF-2 pathway by coimplanting endothelial cells characterized by a widely different activation state (MAECs or pZIPbFGF cells) together with a noncompetent concentration of PGE2 in the rabbit cornea. Capillary formation was observed only in pZIPbFGF im-
planted corneas, whereas none was noted in MAECs or in those receiving PGE2 alone (Figure 3C). Immunohistological analysis at 10 days showed that MAECs and pZIPbFGF cells were alive and no sign of inflammation was present. The simultaneous delivery of PGE2, while having negligible effect on MAECs, greatly potentiated pZIPbFGF cell ability to mount an angiogenic response through the recruitment of resting, limbal, endothelial cells (Figure 3C). These data indicate that the PGE2 proangiogenic activity requires FGF-2 production.

FGFR Is Required in Mediating PGE2 Effects

The above experiments on FGF-2–null cells suggest the involvement of the FGF-2 receptor, FGFR, in the action exerted by PGE2 on the endothelium, because these knock-down cells, although deprived of FGF-2 synthetic machinery, are nonetheless responsive to exogenous FGF-2 (Figure 3B; see also Figure 5B). To examine the FGFR role which leads to FGF-2 production, we challenged CVECs with PGE2 (100 nmol/L) following inhibition of the receptor(s) with SU5402 (10 μmol/L). In this condition, PGE2-induced increase in FGF-2 expression was abolished (Figure 4A).

To further test the hypothesis that PGE2/FGFR/FGF-2 system controls angiogenesis, we used the Matrigel implant assay. Matrigel, either with or without FGF-2, PGE2, SU5402, FGF-2 plus PGE2, or SU5402 plus PGE2 was injected subcutaneously in mice and plugs were harvested.
after 7 days. Implants containing FGF-2 and PGE2 alone or in combination showed several branched structures throughout the implant (Figure 4B). Furthermore, in implants in which PGE2 and SU5402 were concomitantly administered, angiogenesis was reduced, indicating the FGFR(s) requirement for the PGE2 effect. Quantitative analysis of viable/functioning vessels by hemoglobin determination revealed that both FGF-2 and PGE2 alone or in combination produced a nearly 3-fold increase in angiogenesis compared with controls (Figure 4C), whereas administration of SU5402 reduced vessel density by 63% (Figure 4C). Consistently, SU5402 reduced CVECs proliferation promoted by PGE2 (Figure 4D), indicating that PGE2 promotes endothelial cell proliferation by priming the FGF-2 pathway via FGFR activation.

As to the issue of the PGE2 receptor subtype mediating FGFR activation, previously addressed,6 we found that sulprostone (100 nmol/L), administered to endothelial cells, promoted FGF-2 expression, thus confirming the involvement of the EP3 subtype in the PGE2/FGFR signaling (Figure 4E).

Selectivity of PGE2 for FGFR1

Four structurally related tyrosine kinase receptors, denoted FGFR1, FGFR2, FGFR3, and FGFR4,17,18 mediate the biological effects of FGF. We investigated whether FGFR phosphorylation by PGE2 was type selective. In CVECs, the expression of FGFR3 and FGFR4 was undetectable (data not shown), and the prostanoid (100 nmol/L PGE2, for 15 minutes) failed to phosphorylate FGFR2 (Figure 5A). Conversely, FGFR1 was vigorously phosphorylated by the administration of PGE2 and FGF-2 (Figure 5B).

In FGF-2-/- endothelial cells, no FGFR1 phosphorylation occurred at any PGE2 concentration tested, demonstrating that FGFR1 activation by its ligand is a necessary condition for switching the autocrine/paracrine positive amplification cycle, resulting in FGFR1 autophosphorylation (Figure 5B). Tyrosine 766 (Y766) autophosphorylation in the C-terminal domain of FGFR1 is reported to bind and activate the FGFR substrate FRS2α, which in turn stimulates the mitogenic Ras/MEK/MAPK (Ras/mitogen-activated protein kinase/mitogen-activated protein kinase) pathway and

Figure 5. FGFR1 mediates PGE2 activity. A, FGFR2 phosphorylation in CVECs in response to FGF-2 (20 ng/mL) or PGE2 (100 nmol/L; 15 minutes). B, FGFR1 phosphorylation in CVECs and FGF-2-/- cells in response to FGF-2 (0.3 or 20 ng/mL) and/or PGE2 (1 to 100 nmol/L; for 15 minutes). FGFR1 was immunoprecipitated, its activation investigated by anti-P-Tyr antibody, and the results were normalized with total FGFR1. C, FRS2α Y196 phosphorylation in CVECs and FGF-2-/- cells in response to FGF-2 (20 ng/mL or 0.3 ng/mL) and/or PGE2 (1 to 100 nmol/L; 15 minutes). The results were normalized with total FRS2α.
the PLCγ, leading to the release of calcium from intracellular stores. Because PGE2 synergizes with FGF-2/FGFR1 system in promoting proliferation, we evaluated whether the FRS2α phosphorylation mediated the PGE2 mitogenic effects. In both CVECs and FGF-2/−/−, combined PGE2 and FGF-2 (1 nmol/L and 0.3 ng/mL, respectively) promoted robust FRS2α phosphorylation similar to that observed with single compounds at the highest concentrations (100 nmol/L PGE2 or 20 ng/mL FGF-2), whereas none occurred with single compounds at their respective lowest concentration (Figure 5C). In FGF-2/−/− cells PGE2, at any concentration used, failed to phosphorylate FRS2α, indicating the necessity of endogenous FGF-2.

**Downstream Effectors of FGFR1 Modulate PGE2/FGF-2 Synergism**

To delineate the molecular mechanism of PGE2 upregulation of FGF-2 synthesis, we studied extracellular signal-regulated kinase (ERK)1/2 and STAT-3, known effectors of FGF-2 signaling, and eNOS, a potential effector in FGF-2 signaling downstream to FGFR1/PLCγ. Stimulation of CVECs with FGF-2 and/or PGE2 induced PLCγ activation and, in turn, Ca2+-dependent eNOS activation (Figure 6A). FGFR1 activity mediated PLCγ phosphorylation, because SU5402 blunted PGE2 induced response (Figure 6A). Moreover, inhibition of eNOS activity via L-NMMA (200 μmol/L) or silencing eNOS suppressed the FGF-2 expression primed by PGE2 (Figure 6B and 6C). Furthermore, in eNOS/−/− endothelial cells, PGE2 failed to promote FGF-2 expression compared to high serum stimulation (10% CS) (Figure 6D), confirming that eNOS is upstream to FGF-2 production.

Stimulation of CVECs with FGF-2 and/or PGE2 induced ERK1/2 phosphorylation (Figure 6E). The relevance of ERK1/2 activation was demonstrated by its blockade with U0126, a MEK inhibitor, which suppressed FGF-2 production (Figure 6F). Furthermore, PGE2 induced ERK1/2 phosphorylation was partially inhibited by L-NMMA (Figure 6G), indicating that in FGF-2 synthesis pathway, ERK1/2 is in some extent downstream to eNOS activation.

Immunofluorescence experiments in CVECs exposed to PGE2 demonstrated both the phosphorylation and the nuclear translocation of STAT-3 (Figure 7A), a transcription factor known to control FGF-2 expression. In CVECs or
MAECs incubated with PGE2, STAT-3 was dose-dependently phosphorylated at the ERK1/2 dependent phosphorylation site (Figure 7B). Conversely, in pZIPbFGF cells the basal STAT-3 phosphorylation, considerably higher than in MAECs (Figure 7A), was further enhanced by low PGE2 concentration (1 nmol/L), and reduced by higher PGE2 concentrations (Figure 7B).

We also analyzed the sequence of events leading to FGF-2 expression through the activation of FGFR1 and STAT-3. By measuring STAT-3 phosphorylation in response to PGE2 (100 nmol/L) in the presence of a FGFR1 inhibitor (SU5402, 10 μmol/L), we found a nearly complete blockade of STAT-3 phosphorylation (Figure 7C), indicating that FGFR1 activation is required for triggering STAT-3 activation by PGE2.

Confocal microscopy analysis of FGFR1 and STAT-3 localization/activation in response to PGE2 showed marked FGFR1 activation and STAT-3 phosphorylation and translocation to the nucleus (Figure 7D) in CVECs incubated with PGE2 (100 nmol/L). Furthermore, we investigated the link between STAT-3 activation and FGF-2 upregulation in biochemical and functional experiments by using PpYLKTK-mts, a STAT-3 inhibitor. PpYLKTK-mts (25 μmol/L) suppressed both PGE2 induction of FGF-2 overexpression (Figure 7E) and capillary sprouting in mouse aorta explants (Figure 7F, images C and D).

These results show that in activated endothelial cells PGE2 promotes the angiogenic switch through the sequential activation of the autocrine/paracrine FGFRR1/FRS2α/ERK1/2/STAT-3/FGF-2 and FGFR1/PLCγ/cNOs/FGF-2 parallel/interplaying signaling pathways (Figure 8).

**Discussion**

A variety of molecules have been implicated as activators of the angiogenic switch, the triggering event for vessel formation. Among the activators, PGE2, the major COX-2 metabolite, has assumed a major role in promoting the angiogenic switch in tumors, particularly in the development of metastasis. Although the mechanism that executes the PGE2 angiogenic program in the endothelium is poorly understood, previous observations have shown a tight interdependence between PGE2 ability to exert its angiogenic effect and the activation of the autocrine/paracrine FGF-2/FGFR1 signaling pathway in endothelial cells. Here, we report studies that provide a mechanistic interpretation of the angiogenic switch promoted by PGE2 in the endothelium.

The major finding of this study is the existence of a remarkable synergism between the PGE2 and the FGF-2 pathways. This was shown both in cultured endothelial cells and in vivo, in the rabbit cornea angiogenic assay and in Matrigel implants in mice. In both conditions, PGE2 and FGF-2, applied concomitantly at noncompetent doses, elicited angiogenic responses far above those attained by the single molecules. That the synergic interaction might be the functional underpinning for the angiogenic switch triggered by the prostanoid is supported by several lines of evidence. First, we found that PGE2 induction of endothelial cell

![Figure 7. PGE2 activates STAT-3. A, Effect of PGE2 on STAT-3 phosphorylation and nuclear translocation. After 15 minutes of stimulation with PGE2 (100 nmol/L) and FGF-2 (20 ng/mL), cells were labeled for phospho–STAT-3 and observed at fluorescence microscopy. Original magnification, ×40. B, STAT-3 phosphorylation induced by PGE2. pZIPbFGF, MAECs, and CVECs were treated with PGE2 (1 to 100 nmol/L; 15 minutes), and then STAT-3 phosphorylation was investigated by Western blotting. C, CVECs pretreated with SU5402 (10 μmol/L; 30 minutes) were stimulated with PGE2 (15 minutes). STAT-3 phosphorylation evaluated by Western blotting. D, Confocal microscopy of FGFR1 localization and STAT-3 activation on endothelial cells (CVECs). Control cells (1, 2, and 3) or PGE2-treated (4, 5, and 6) and FGF-2–treated (7, 8, and 9) cells were analyzed for FGFR1 localization (1, 4, and 7) and STAT-3 phosphorylation (2, 5, and 8). E, FGF-2 expression following STAT-3 inhibitor treatment (PpYLKTK-mts, 25 μmol/L). F, Vessel sprouting from mouse aorta rings at day 4 of incubation: control (1% serum) (A), PGE2 (B), STAT-3 inhibitor (C), PGE2 in the presence of the STAT-3 inhibitor (D). F, Sprouting area is expressed as the number of grid units (NGU) covering the entire surface occupied by pseudocapillaries (n=3). Experiments were run in duplicate.](http://circres.ahajournals.org/content/664/9/5679/F7.large.jpg)
pGL3 promoter plasmid, respectively,14,15 was induced by combined concentrations of PGE$_2$/FGF-2, whereas no phosphorylation was found in FGF-2–null cells. In addition, inhibiting eNOS activity or suppressing its expression as well, blocking ERK1/2 or STAT-3 activation, inhibited the activation of the autocrine/paracrine FGF-2/FGFR1 cycle and blocked PGE$_2$ effect on endothelial cell proliferation and capillary sprouting from aorta explants.

Proof that the synergic interaction between PGE$_2$ and FGF-2 occurs in vivo is provided by the strikingly high angiogenic responses in rabbit corneas in which cells over-expressing FGF-2 (pZIPbFGF) were implanted in combination with a noncompetent dose of PGE$_2$. The strong capillary growth in Matrigel implants in mice further substantiates these findings. These data and particularly those obtained with FGF-2–null cells underscore the respective role of FGF-2/FGFR and PGE$_2$, the relationship being one of necessity (FGF-2) versus sufficiency (PGE$_2$).

Among the 4 known FGF receptors, FGFR3 and FGFR4 were not detectable in our cells, as well as in other endothelial cells.27 Because we could not detect FGFR2 phosphorylation by PGE$_2$, we propose that the prostanooid specifically activates FGFR1, at least under our experimental conditions. Beside the FGFRs, a 4-member family of heparan sulfate proteoglycans, the syndecans are important players in FGF signaling. Syndecan-4, shown to increase the FGF-2 affinity for FGFR1, may be also a suitable candidate in our system, particularly in the observed PGE$_2$ regulation of eNOS and FGFR1.28,29

Consistent with the previous report, the PGE$_2$ receptor subtype involved in this effect was shown to be EP3, a subtype widely expressed in vascular endothelium30 and characterized by the highest affinity for PGE$_2$. It is noteworthy that neither vascular endothelial growth factor nor epidermal growth factor, well-known angiogenic factors, synergized with PGE$_2$ in promoting endothelial activation.

Conceivably, this synergistic mechanism may function in several pathophysiological settings, such as tissue healing, collateralization, revascularization, and smoldering inflammation, in which both PGE$_2$ and FGF-2 are released in the microenvironment.31–33 In the light of our findings, we propose a model for the angiogenic switch based on the autocrine/paracrine FGF-2/FGFR1 activation by PGE$_2$ and FGF-2 synergistic interaction. FGFR1 acts as the master switch, because its activation by FGF-2 initiates an autocrine cycle of FGF-2 synthesis and FGFR1 activation. PGE$_2$ acts as a primer of the angiogenic switch by promoting the FGF-2/FGFR1 interaction through upregulation of FGF-2 expression and mobilization from the extracellular matrix. Multiple checkpoints are likely to be operating in the proposed mechanism, as examples, desensitization of the EP3 receptor and/or its intracellular signals, enzymatic degradation of PGE$_2$, or feedback mechanisms working at the level of the transcription factor STAT-3.

In conclusion, the synergism between the PGE$_2$ and FGF-2 signaling pathways here described may explain the mechanism of action of drug combinations, the most notable being COX inhibitors with growth factors or growth factor receptor inhibitors,34–36 and provide the basis for a precise targeting of molecules involved in disease initiation and progression.

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Disclosures

None.

References


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