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Prostaglandin E_2 Regulates Angiogenesis via Activation of Fibroblast Growth Factor Receptor-1*[S]

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Prostaglandin E_2 (PGE_2) behaves as a mitogen in epithelial tumor cells as well as in many other cell types. We investigated the actions of PGE_2 on microvascular endothelial cells (capillary venular endothelial cells) with the purpose of delineating the signaling pathway leading to the acquisition of the angiogenic phenotype and to new vessel formation. PGE_2 (100 nM) produced activation of the fibroblast growth factor receptor 1 (FGFR-1), as measured by its phosphorylation, but not of vascular endothelial growth factor receptor 2. PGE_2 stimulated the EP3 subtype receptor, as deduced by abrogation of EP3 $G\alpha_i$ subunit activity through pertussis toxin. Consistent with this result, in human umbilical venular endothelial cells missing the EP3 receptor, PGE_2 did not phosphorylate FGFR-1. Upon binding to its receptor, PGE_2 initiated an autocrine/paracrine signaling cascade involving the intracellular activation of c-Src, activation of matrix metalloproteinase (predominantly MMP2), which in turn caused the mobilization of membrane-anchored fibroblast growth factor-2 (FGF-2). In fact, in cells unable to release FGF-2 the transfection with both FGFR-1 and EP3 did not result in FGFR-1 phosphorylation in response to PGE_2. Relevance for the FGF2-FGFR-1 system was highlighted by confocal analysis, showing receptor internalization after cell exposure to the prostanoid. ERK1/2 appeared to be the distal signal involved, its phosphorylation being sensitive to either cSrc inhibitor or FGFR-1 blocker. Finally, PGE_2 stimulated cell migration and capillary formation in aortic rings, which were severely reduced by inhibitors of signaling molecules or by receptor antagonist. In conclusion, this study provides evidence for the involvement of FGFR-1 through FGF2 in eliciting PGE_2 angiogenic responses. This signaling pattern is similar to the autocrine-paracrine mechanism which operates in endothelial cells to support neovascular growth.

Chronic inflammation is a critical component of tumor progression. Prostaglandin E_2 (PGE_2),[2] once viewed as the prototypical mediator of inflammation, is now regarded as a promoter of neoplastic growth and of tumor angiogenesis. This notion has been established by experimental studies which show increased expression of cyclooxygenase isoforms (COX-1 and COX-2) as well as enhanced levels of PGE_2, the major product of their enzyme activity, in several tumor tissues (*i.e.* colon and breast tumors) (1–4).

A wealth of experimental studies has delineated the molecular mechanisms utilized by PGE_2 to induce tumor proliferation. PGE_2 upon binding to its membrane receptor, belonging to the classical G protein-coupled receptor family, activates a signal cascade that through a complex array of intermediate steps (c-Src, PKC, Pyk2), leads to the extracellular release of peptide ligands stimulating growth factor receptors and producing tumor growth (5). In parallel, PGE_2 transactivates the EGF receptor (EGFR) via an intracellular phosphorylation cascade involving the protooncogene c-Src, which magnifies the EGF tumorigenic drive (6–8).

Complementing its tumor promoting activity, PGE_2 has been found to activate tumor angiogenesis, thus providing for the blood supply needs of proliferating tumors (1). Experimental evidence shows that the overexpression of cyclooxygenase-2, which characterizes many epithelial tumors as well as their endothelial population, is accompanied by enhanced expression and production of angiogenic factors such as vascular endothelial growth factors (VEGF), fibroblast growth factor-2 (FGF-2), hypoxia-inducible factor-1, matrix-degrading enzymes (*e.g.* matrix metalloproteinases (MMPs)), vascular remodeling ligands (*i.e.* angiopoietins), and adhesion receptors of the integrin families (9–15). Indeed, it appears that in experimental breast tumors, characterized by high output of PGE_2 through forced expression of cyclooxygenase-2, angiogenesis proceeds tumor development (16). Thus, the concept that PGE_2 is capable to drive tumor angiogenesis is now firmly grounded. Numerous clinical investigations conducted either as population studies or specific investigations on nonsteroidal anti-inflammatory drugs, which produce a decrease of PGE_2 tissue level through blockade of cyclooxygenase-2, have further established the crucial role of prostanoid in angiogenesis, since most angiogenic markers examined were definitely down-regulated, and tumor progression was halted (1).

Although the action of PGE_2 on tumor-associated angiogenesis appears well documented, much less is known about the

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[2] The abbreviations used are: PGE_2, prostaglandin E_2; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; EGFR, EGF receptor; CVEC, capillary venular endothelial cells; HMVEC-C, human microvascular endothelial cells; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; FGF, fibroblast growth factor; FGFR, FGF receptor; MMP, metalloproteinase; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline.

activity of the prostanoid on the angiogenic process outside of the context of tumor growth. Previous reports indicated that PGE_2 induces directly endothelial cell proliferation, migration, and tube formation (17, 18). In particular it has been demonstrated that PGE_2 induces angiogenesis after activation of EP2 and EP4 receptors in human vascular endothelial cells (17, 18). EP2 or EP4 activation induces an increase of intracellular levels of cAMP and ERK1/2, linked to different signaling pathways. Recently, Rao *et al.* (18) demonstrated that EP4 receptor plays a critical role in PGE_2-dependent *in vitro* migration and tubulogenesis that is mediated by activation of ERK1/2 pathway. Moreover, Kamoshita *et al.* (19) indicated in EP3 receptor the subtype involved in wound healing and angiogenesis. Finally, Namkoong *et al.* (17) demonstrated that pharmacological inhibition of endothelial nitric-oxide synthase (eNOS) pathway inhibits PGE_2-induced proliferation in *in vitro* cultured endothelial cells and that in eNOS-deficient mice the formation of capillary-like structure on aortic rings is abolished.

Moreover, FGF-2 and VEGF, two effective pro-angiogenic growth factors, induce angiogenesis through increasing expression of cyclooxygenases and PGE_2 production (20, 21). However, the functional and molecular mechanisms necessary for PGE_2-induced angiogenesis are not completely understood.

In this work we have investigated the action of PGE_2 on microvascular endothelial cells examining both their functional properties relevant for angiogenesis (migration and ability to form pseudocapillaries) and signals involved in producing the angiogenic phenotype, such as activation of ERK1/2 and of the growth factor receptor, FGFR-1.

EXPERIMENTAL PROCEDURES

Cell Line and Culture Conditions—Post-capillary venular endothelial cells (CVEC) were obtained and cultured as previously described (22). Cardiac-derived human microvascular endothelial cells (HMVEC-C) and human umbilical venular endothelial cells were purchased from Cambrex and maintained in Endothelial Cell Basal Medium-2 with growth supplements. Chinese hamster ovary cells transfected with FGFR-1 (CHO/FGFR-1) were provided by Prof. M. Presta (University of Brescia, Brescia, Italy) and cultured as previously described (23).

Cell Migration—Chemotaxis experiments were performed with the Boyden chamber technique as previously described (24). Endothelial cells were treated for 30 min with U0126 (10 nM), PP1 (500 nM), GM6001 (25 μM), or SU5402 (10 μM), and then 1.25×10^4 cells were added to the upper wells of the chamber. PGE_2 (100 nM) was used as chemoattractant.

Western Blot—Cells (3×10^5) were seeded in 60-mm-diameter dishes. Cells were stimulated with 100 nM PGE_2. To assess the effects of FGFR1 and c-Src inhibitors on ERK1/2 phosphorylation, cells were pretreated with SU5402 (10 μM) or PP1 (500 nM). To evaluate the expression of EP receptors in CHO-FGFR1 transfected with EPs, 8×10^5 cells were seeded in 100-mm-diameter dishes, and after 24 h cells were analyzed by Western blotting. Western blot was performed as previously described (25).

Immunoprecipitation—Cells were stimulated with PGE_2 (100 nM) or FGF-2 (20 ng/ml) for 15 min. Where indicated, cells were pretreated with the anti-FGF-2 neutralizing antibody (6 μg/ml), PP1 (500 nM), GM6001 (25 μM), or MMP-2 inhibitor (25 μM) for 30 min and with pertussis toxin (300 ng/ml) for 18 h. EP receptor agonists (100 nM), adenosine 3′,5′-cyclic monophosphate, N^6,O_2 dibutyryl-sodium salt (cAMP analogue, 10 μM), or A23187 (50 ng/ml) were added to the cells for 15 min. Anti-FGFR-1 or anti VEGFR-2 antibody were added to the precleared lysates (100 μl, Sigma). Western blot was performed as previously described (25).

Gelatin Zymography—6×103 cells/well were cultured in 96-well cell culture plates in 10% fetal calf serum medium. After adhesion, cells were washed with and incubated in serum-free medium for 18 h. 100 nM PGE_2 were added in 50 μl of fresh serum-free medium. After 18 h of incubation, the conditioned medium was collected, clarified by centrifugation, and assayed for zymography as described (25).

Immunohistochemistry—25×10^3 CVEC cells were seeded on cover slides in a 24-multiwell plate. Cells were starved for 24 h and then stimulated with FGF-2 20 ng/ml or PGE2 100 nM for 15 min. After the stimulation the cells were fixed in paraformaldehyde for 5 min and then washed in PBS with Ca^{2+} and Mg^{2+}. Cells were then permeabilized in 0.25% Tween 20 in PBS for 10 min. After the blocking of unspecific bindings in 3% bovine serum albumin (BSA) for 30 min, the cells were incubated with a monoclonal mouse anti FGFR1 antibody (Upstate) diluted 1:25 in 0.5% BSA in PBS for 18 h at 4 °C. Cells were then washed and incubated with a goat fluorescein isothiocyanate anti-mouse Ig G (Sigma) diluted 1:100 for 1 h. The cells counterstained with propidium iodide (1.5 mM) were also pretreated with 100 μg/ml DNase-free RNase.

Heparin Binding—10 μl of heparin-acrylic beads were incubated with increasing concentration of PGE_2 (100 nM to 10 μM) in 40 μl of PBS for 1 h at 37 °C. Then 25 ng of FGF-2 was added and incubated for 2 h at 37 °C. Beads were then washed with PBS, and bound FGF-2 was removed by boiling with sample buffer. The solution was analyzed by SDS, 8% polyacrylamide gel. Western blots for FGF-2 were performed as described (26).

Cell Transfection—Subconfluent CHO-FGFR-1 were incubated in Dulbecco's modified Eagle's medium, 1% fetal calf serum with 6 μg of prostaglandin E receptor 1, 2, 3, or 4 subtype encoding plasmids or empty plasmid (pcDNA3.1) and 6 μl of Lipofectamine for 18 h and then cultured in fresh medium for 24 h before use in experiments.

Endothelial Tube Formation from Aortic Rings—Pseudocapillary sprouting from vessel rings was evaluated as previously described (25). Stimuli were tested at 20 ng/ml FGF-2 or 100 nM PGE_2. In experiments aimed at evaluating the role of FGFR-1, the selective inhibitor SU5402 (10 μM) was added together with PGE_2. The area occupied by pseudocapillary structures was quantified by an inverted microscope at a magnification of 200× using an ocular grid. The area is expressed as the number of grid units required to cover the entire pseudocapillary surface.

Reagents—Reagents for cell cultures, PGE_2, misoprostol, heparin-acrylic beads, pertussis toxin, A23187, and heparinase were obtained from Sigma; U0126, SU5402, SU5614, PP1 GM6001, and MMP-2 inhibitor-I were from Calbiochem-Novabiochem; FGF-2 was from Peprotech. Anti-phospho-

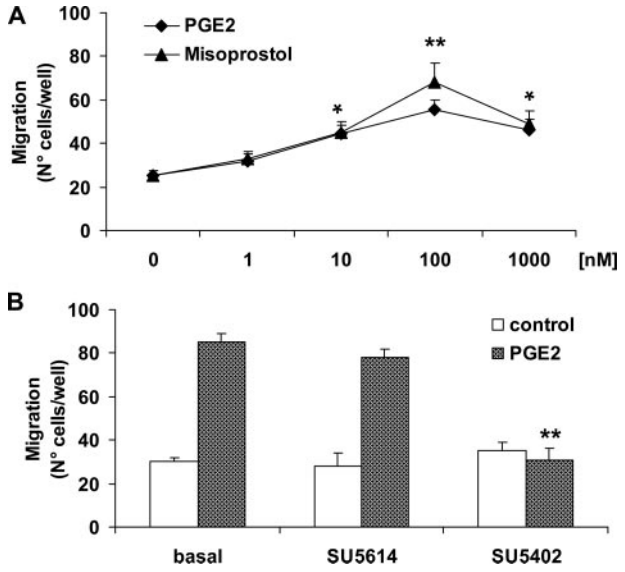

FIGURE 1. **PGE_2 induces endothelial cells migration through FGFR-1 activation.** *A*, sparse, synchronized endothelial cells were exposed to PGE_2 or Misoprostol (1–1000 nM). Data are reported as cell number counted/well. ($n = 4$). $p < 0.01$ (*) and $p < 0.001$ (**) *versus* 0.1% serum. *B*, CVEC were treated with SU5614 (10 μM) or SU5402 (10 μM), then stimulated with PGE_2 (100 nM) for 4 h. Cell migration was measured as cell number counted/well. **, $p < 0.001$ *versus* PGE_2 alone ($n = 3$).

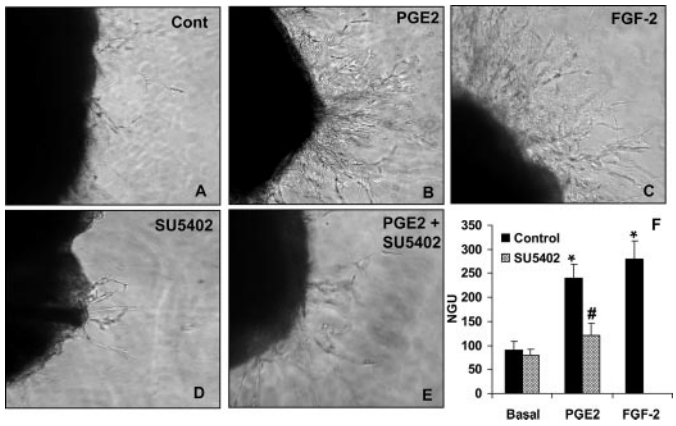

FIGURE 2. **Blockade of vessel formation by FGFR-1 tyrosine kinase inhibitor SU5402.** Capillary sprouting from mouse aorta rings quantified at day 4 as the area covered by new capillaries. $n = 3$ experiments run in duplicate. Representative pictures of vessel sprouting. *A*, control (1% serum). *B*, PGE_2-induced vessel formation. *C*, FGF-2-induced vessel formation. *D*, SU5402 stimulation. *E*, SU5402 and PGE_2 stimulation. *F*, pseudocapillary formation was monitored by inverted microscope at a 10× magnification using an ocular grid. The area covered by pseudocapillary structures was expressed as the number of grid units (*NGU*). *, $p < 0.01$ *versus* basal; #, $p < 0.01$ *versus* PGE_2 alone.

ERK1/2, anti-ERK1/2, and anti-phosphotyrosine antibodies were purchased from Cell Signaling; anti-FGF-2 neutralizing, anti-FGFR-1, or anti-VEGFR-2, and anti-FGF-2 antibodies were from Upstate; anti-β-actin was from Sigma. Plasmids encoding for EP1, EP2, EP3, or EP4 receptors (PTGER1, PTGER2, PTGEE3, and PTGER4) were from UMR cDNA Resource Center, University of Missouri-Rolla.

Statistics—Statistical analysis was performed using Student's *t* test for unpaired data or by analysis of variance; $p < 0.05$ was considered statistically significant.

RESULTS

Prostaglandin E_2 Induces Endothelial Cell Migration and New Vessel Formation through FGFR-1 Activation—The pro-angiogenic properties of PGE_2 have been documented in several reports describing its effects either on the *in vivo* formation of new vessels or on cultured endothelial cells measuring functional responses (proliferation, migration) (17, 18, 20, 27, 28). This work focuses on signaling mechanisms underlying the angiogenic actions exerted by PGE_2 on cultured microvascular endothelial cells. First, we analyzed the chemotactic activity of PGE_2 by measuring cell migration after incubation of quiescent endothelial cells with increasing concentrations of the prostanoid (1–1000 nM for 4 h). The observed response to PGE_2 was concentration-related, maximal effect being reached at 100 nM (a concentration used throughout this work). Misoprostol, a metabolically stable PGE_2 analogue, reproduced the effect of the natural ligand, suggesting that PGE_2 is stable under the conditions used, and its effects may not be attributed to its derivative products (Fig. 1*A*). In light of these results we wondered whether PGE_2 activity might involve canonical pathways of angiogenesis such as those elicited by the FGF-2 or the VEGF. We, therefore, measured PGE_2-induced cell migration in the presence of antagonists of their respective receptors (FGFR-1 and VEGFR-2). Indeed, FGFR-1 blockade by SU5402 (10 μM) abolished cell migration, whereas application of SU5614 (10 μM), a blocker of VEGFR-2, did not modify PGE_2 action (Fig. 1*B*).

The PGE_2 pro-angiogenic activity was also analyzed in mouse aorta explants, a vascular organ culture representing an integrated system for assessing angiogenesis. As shown in Fig. 2, PGE_2 produced the formation of a rich network of capillary-like structures in mouse aorta explants. The magnitude of the PGE_2 response was comparable with that promoted by the angiogenic factor FGF-2 (Fig. 2, *A*, *B*, *C*, and *F*). SU5402, (10 μM), the selective FGFR-1 tyrosine kinase inhibitor, abolished PGE_2-induced capillary sprouting (Fig. 2, *D–F*), whereas SU5614, the VEGFR-2 inhibitor, was devoid of any effect (not shown). To investigate the selectivity of FGFR-1 activation by PGE_2, mouse aortic explants were pretreated with SU5402 and stimulated with selective thromboxane-A and PGI_2 agonists (U4699 and Iloprost at 100 nM, respectively). Iloprost induced the formation of capillary-like structures, which were unaffected by FGFR-1 inhibition, whereasU4699 was not able to promote neovessel growth in this experimental model (data not shown). Collectively, these findings clearly indicate that FGFR-1 activation selectively conveys PGE_2 mitogenic signals in the vascular endothelium, promoting its activation and migratory behavior.

PGE_2 Promotes FGFR-1 Activation—To determine the direct involvement of FGFR-1 in the above described responses, we examined whether PGE_2 could stimulate its phosphorylation. Activation of FGFR-1 was evaluated by the immunoprecipitation of the receptor followed by Western blotting analysis with phosphotyrosine-specific antibody. PGE_2 treatment of endothelial cells induced rapid FGFR-1 phosphorylation (2-fold increase over basal), detectable within 10 min (not shown) and sustained through 15 min of incubation (Fig. 3*A*). FGF-2 at a fully competent concentration of 20 ng/ml produced a slightly more intense FGFR-1 phosphorylation (3-fold over basal).

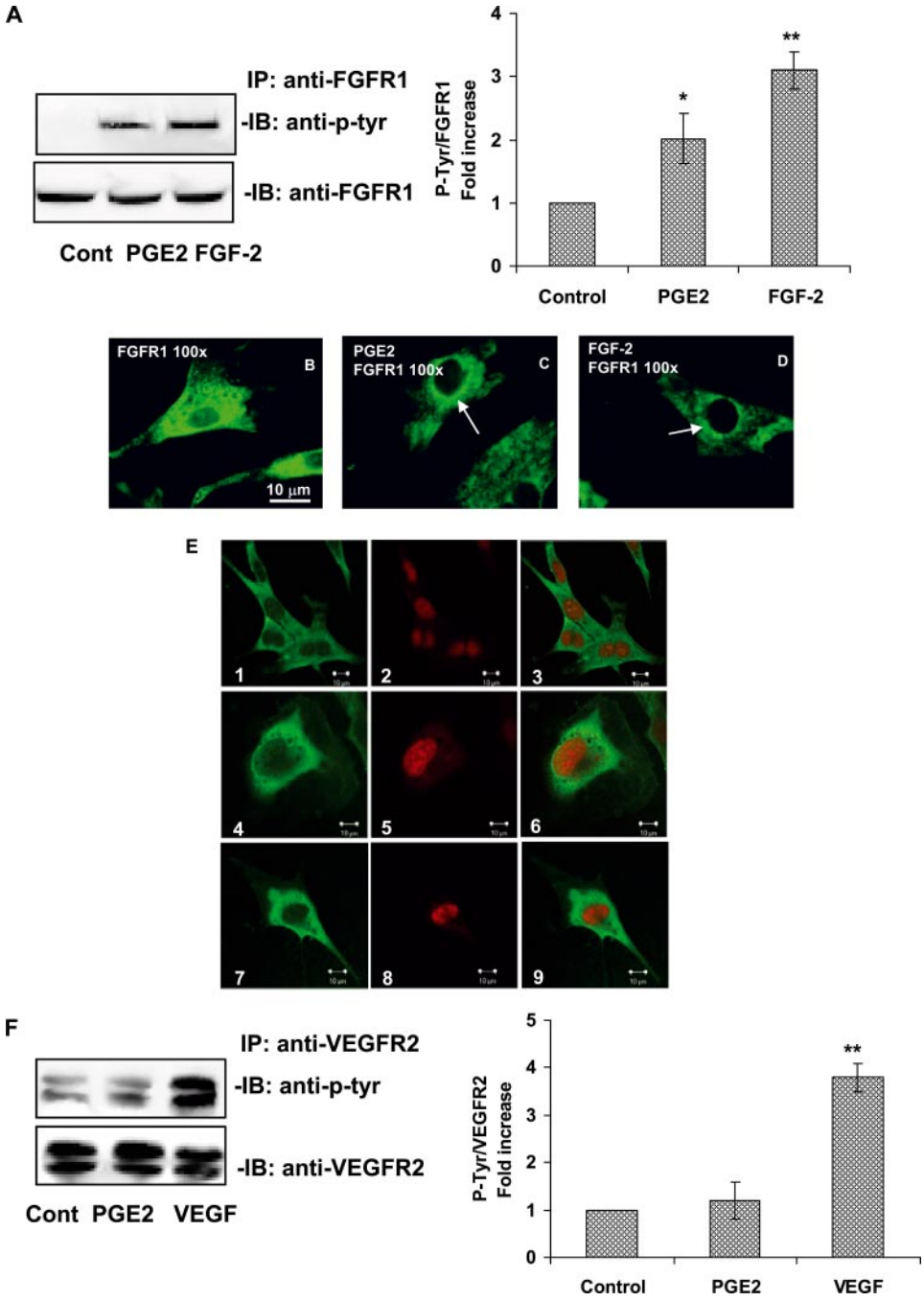

FIGURE 3. **PGE_2 induces FGFR-1 phosphorylation and nuclear translocation.** *A*, 100 nM PGE_2 induced FGFR1 phosphorylation in endothelial cells (CVEC). FGFR-1 was immunoprecipitated (*IP*), and its activation has been investigated by anti-Tyr antibody. Results were normalized with FGFR-1. The gels shown are representative of three obtained with similar results. *Cont*, control. *IB*, immunoblot. *B*, immunofluorescence analysis of FGFR-1 localization in endothelial cells (CVEC). *C* and *D*, effect of PGE_2 (100 nM) and or FGF-2 (20 ng/ml) stimulation on FGFR-1/FGF-2 translocation in the perinuclear membrane. Original magnification, 100×. *E*, confocal microscopy of FGFR-1 localization in endothelial cells (CVEC). In control (*1*, *2*, and *3*) or PGE2 (*4*, *5*, and *6*)- and FGF-2 (*7*, *8*, and *9*)-treated cells immunohistochemical analysis for FGFR-1 localization was performed (*1*, *4*, and *7*). Nuclei were counterstained with propidium iodide (*2*, *5*, and *8*). *F*, PGE_2 doesn't induce VEGFR-2 phosphorylation. VEGFR-2 was immunoprecipitated, and its activation has been investigated by anti-Tyr antibody. Results were normalized with anti-VEGFR-2 antibody.

We confirmed this finding by using a different approach. We studied the perinuclear translocation of FGFR-1 in response to PGE_2, a known mechanism linked to tyrosine kinase receptor activation (29). The addition of PGE_2 to the medium promoted within minutes (15 min) translocation of FGFR-1 from the membrane/cytoplasm to the perinuclear area (Fig. 3*C*), thus reproducing the receptor internalization observed with the natural ligand FGF-2 (Fig. 3*D*). To better analyze this phenomenon we used confocal microscopy using a nuclear marker (propidium iodide). As shown in Fig. 3*E*, PGE_2 or FGF-2 resulted in internalization of FGFR-1 in perinuclear area (see *panel 3 versus 6* and *9*). FGFR-1 activation was clearly specific, as the prostanoid failed to promote VEGFR-2 phosphorylation (Fig. 3*F*).

Phosphorylation of FGFR-1 by PGE_2 Requires the Activation of c-Src—To delineate the mechanism of FGFR-1 activation, we first investigated whether PGE_2 would directly bind to the receptor by using CHO cells overexpressing FGFR-1 but lacking EP receptors. Exposure of CHO/FGFR-1 to PGE_2 failed to induce FGFR-1 phosphorylation (Fig. 4*A*).

Because PGE_2 has been reported to activate c-Src in tumor cells and c-Src has been shown to serve as a signaling mediator both downstream and upstream of growth factor receptor activation (7, 30), we determined whether PGE_2 activates c-Src in endothelial cells and whether its activation lies upstream of the FGFR-1. PGE_2 promoted a robust (nearly 4-fold increase over basal) c-Src phosphorylation (Fig. 4*B*). We then evaluated the influence of c-Src on the PGE_2-induced FGFR-1 activation by measuring its phosphorylation in the presence of PP1, a known c-Src inhibitor, in comparison to that of SU5402. Because both compounds inhibited FGFR-1 phosphorylation to a similar extent, we conclude that PGE_2 activates FGFR-1 through a c-Src-dependent mechanism that appears to be upstream to the growth factor receptor activation (Fig. 4*C*).

PGE_2 Activates FGFR-1 through an FGF-2 Mobilization Mechanism—We then examined the possibility that the prostanoid might act through the extracellular shedding of FGF-2 by the sequential stimulation of c-Src and matrix metalloproteinases.

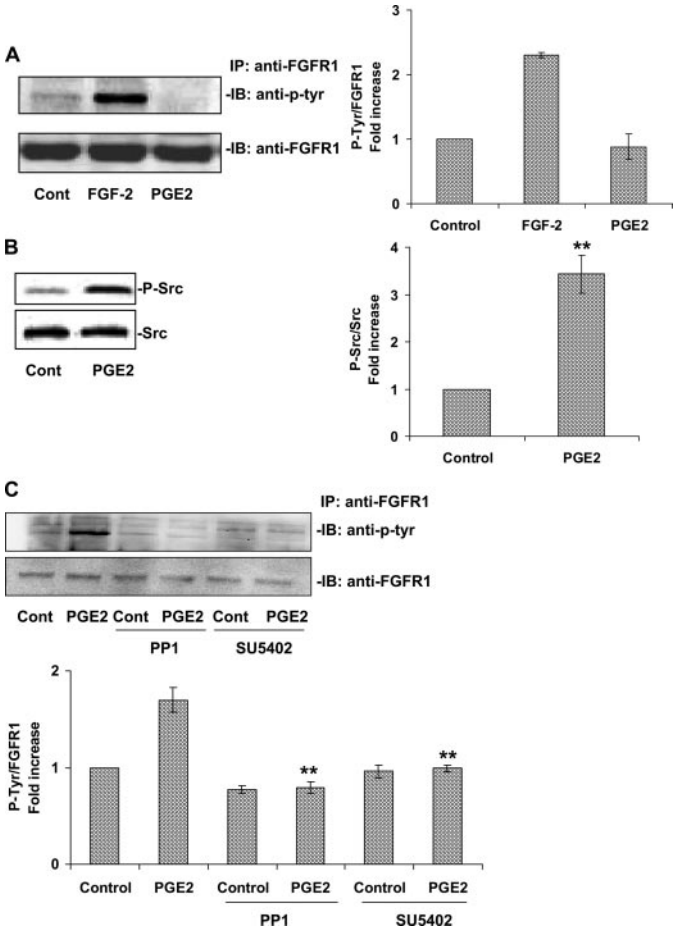

FIGURE 4. **PGE_2 promotes FGFR-1 phosphorylation through c-Src.** *A*, FGFR1 phosphorylation in CHO/FGFR-1 cells in response to FGF-2 (20 ng/ml) and or PGE_2 (100 nM) after 15 min of stimulation. FGFR-1 was immunoprecipitated (*IP*), its activation was investigated by anti-Tyr antibody, and the results are normalized with FGFR-1. The gels shown are representative of three obtained with similar results. *IB*, immunoblot. *B*, 100 nM PGE_2 induced Src phosphorylation. Results were normalized with actin. The gels shown are representative of three obtained with similar results. *C*, FGFR-1 phosphorylation in endothelial cells CVEC in response to PGE_2 (100 nM) in the presence or absence of the Src inhibitor PP1 (500 nM) or the FGFR-1 inhibitor SU5402 (10 μM). FGFR-1 was immunoprecipitated, its activation was investigated by anti-Tyr antibody, and results normalized with FGFR-1. The gels shown are representative of three obtained with similar results. *Cont*, control.

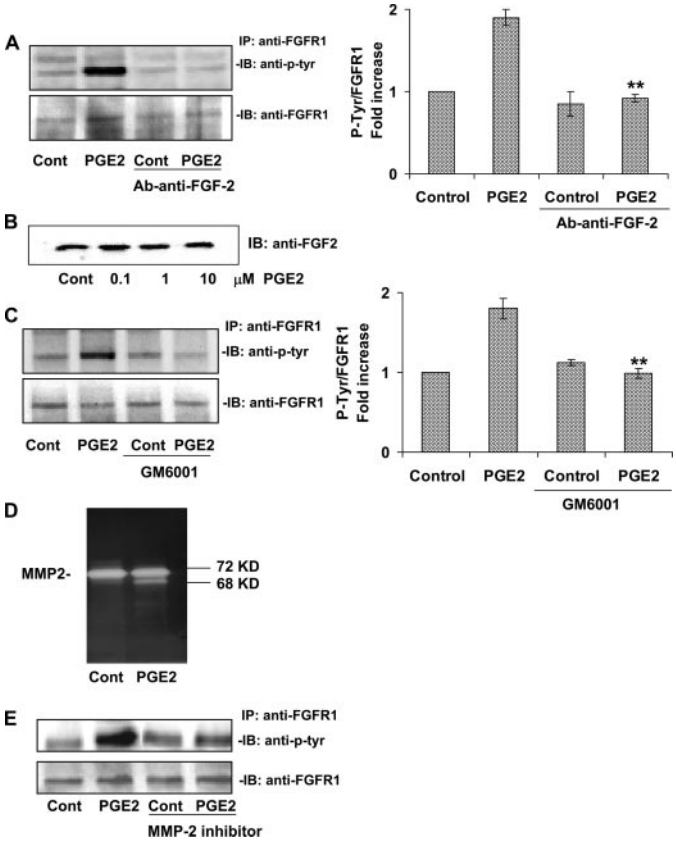

FIGURE 5. **PGE_2 activates FGFR-1 through an FGF-2 mobilization mechanism.** *A*, neutralizing antibody anti-FGF2 (*Ab*) blocks PGE_2-induced FGFR-1 phosphorylation. CVEC have been were pretreated for 30 min with antibody (6 ng/ml) and then exposed to PGE_2 (100 nM) for 15 min. FGFR-1 was immunoprecipitated (*IP*), and its activation has been investigated by anti-Tyr antibody. Results were normalized with FGFR-1. *Cont*, control. *IB*, immunoblot. *B*, FGF-2 heparin binding was not affected by PGE_2. Acrylic heparin beads were treated with PGE_2 from 0.1 to 10 μM for 1 h at 37 °C, then incubated with FGF-2 (25 ng). Bound FGF-2 was recovered and revealed by Western blot. (n = 3). *C*, MMP inhibitor GM6001 inhibits PGE_2-induced FGFR1 activation. CVEC have been pretreated for 30 min with GM6001 and then treated with PGE_2 100 nM for 15 min. FGFR-1 was immunoprecipitated, and its activation was investigated by anti-Tyr antibody. Results were normalized with FGFR-1. The gels shown are representative of three obtained with similar results. *D*, CVEC were treated with 100 nM PGE_2 for 18 h, and then the MMP-2 activation was evaluated by zymography. The gels shown are representative of two obtained with similar results. *E*, FGFR-1 phosphorylation in response to PGE_2 (100 nM) in the presence or absence of the MMP-2 inhibitor (25 μM). FGFR-1 was immunoprecipitated, its activation investigated by anti-Tyr antibody, and the results are normalized with FGFR-1. The gels shown are representative of three obtained with similar results.

To determine whetherFGF-2 might have a role in the activation of FGFR-1 by PGE_2, we used a non-permeant neutralizing antibody for FGF-2 and measured the phosphorylation of FGFR-1 in response to PGE_2. Application of the antibody to endothelial cells 30 min before challenge with PGE_2 (15 min) fully prevented its ability to phosphorylate FGFR-1 (Fig. 5*A*). We further explored the possibility that PGE_2 might displace FGF-2 from its storage sites by disrupting its binding to heparin on cell membranes (31). The observation that FGF-2 binding to heparin-coated beads is unperturbed by the presence of PGE_2 negates this hypothesis (Fig. 5*B*) and indicates that PGE_2 does not directly affect the FGF-2 binding to its intermediate storage site.

To reveal the mechanistic effect of PGE_2 on FGF-2 shedding, we examined whether the prostaglandin induced FGF-2 mobilization by MMP activation. The relevance of MMP activity in the process of FGFR-1 phosphorylation was investigated by using a potent MMP inhibitor (GM6001 25 μM, 30 min) before PGE_2 challenge (15 min). Because GM6001 suppressed FGFR-1 phosphorylation (Fig. 5*C*), we deduce that PGE_2 induces MMP activation to promote the mobilization of FGF-2 from membrane stores. Thus, mobilized-FGF-2 promotes FGFR-1 phosphorylation through an autocrine/paracrine system.

We also identified the MMP involved in FGF-2 mobilization. Zymography analysis indicated that PGE_2 promotes MMP-2 production/activation (Fig. 5*D*). Consistent with this result, MMP-2 inhibition (25 μM, 30 min of pretreatment) significantly reduced FGFR-1 phosphorylation (Fig. 5*E*), suggesting that this metalloproteinase is the major player in FGF-2 shedding.

EP3 Receptor Subtype Promotes FGFR-1 Phosphorylation—We also studied the receptor subtype involved in PGE_2-induced

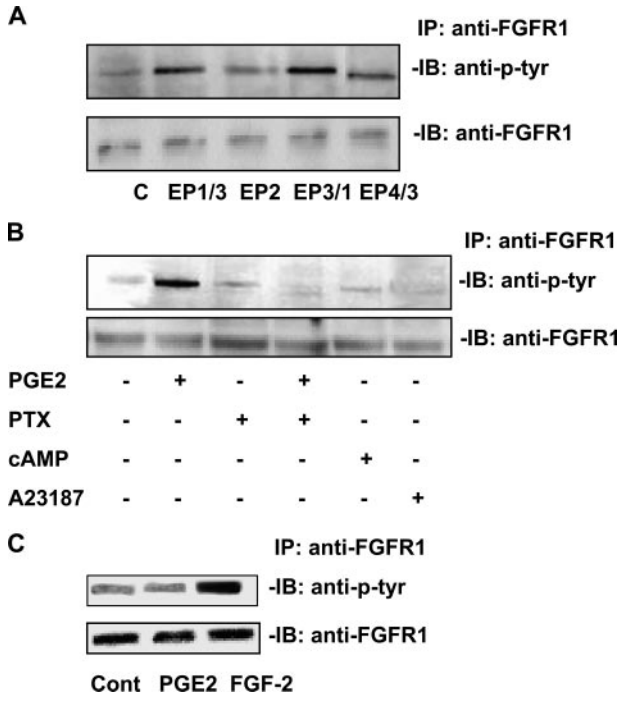

FIGURE 6. **EP3 receptors mediate FGFR1 activation.** *A*, FGFR-1 phosphorylation induced by agonists of the EP receptor subtypes (EP1/3, 17-phenyl trinor prostaglandin E-2; EP2, Butaprost; EP3/1, sulprostone; EP4/3, prostaglandin E-1 alcohol, all used at 1 μM) after 15 min stimulation. The gel is representative of two with similar results. *IP*, immunoprecipitate; *IB*, immunoblot. *B*, CVEC were pretreated with pertussis toxin (*PTX*, 300 ng/ml) for 18 h and then stimulated with PGE_2 (100 nM), cAMP analogous (10 μM), or calcium ionophore A23187 (50 ng/ml) for 15 min. FGFR-1 was immunoprecipitated, and its activation has been investigated by anti-Tyr antibody. Results were normalized with FGFR-1. The gels shown are representative of two obtained with similar results. *C*, human umbilical venular endothelial cells were treated with 100 nM PGE2 or 20 ng/ml FGF-2 for 15 min. FGFR-1 was immunoprecipitated, and its activation has been investigated by anti-Tyr antibody. Results were normalized with FGFR-1. The gels shown are representative of four obtained with similar results. *Cont*, control.

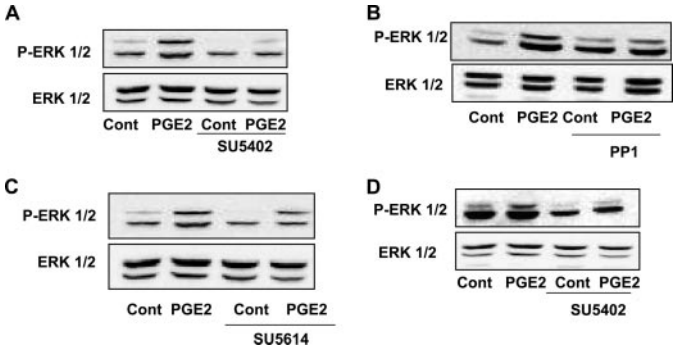

FIGURE 7. **ERK1/2 phosphorylation requires FGFR-1 activation.** CVEC (*A*) and HMVEC-C (*D*) were treated with SU5402 (10 μM), then stimulated with PGE_2 (100 nM) for 15 min, and ERK1/2 activity was measured. *B* and *C*, CVEC were treated with SU5614 (10 μM) or PP1(500 nM), respectively, then stimulated with PGE_2 (100 nM) for 10 min, and ERK1/2 activity was measured by Western blotting analysis. Results were normalized with total ERK1/2. The gels shown are representative of three obtained with similar results. *Cont*, control.

FGFR-1 phosphorylation. We examined whether agonists for the individual subtype were able to phosphorylate FGFR-1. Stimulation with 100 nM EP1/3, EP3/1, and EP3/4 agonists produced consistent phosphorylation of FGFR-1 (Fig. 6*A*), whereas EP2 agonist was devoid of effect. The extent of phosphorylation was clearly more marked for agonists activating EP3 receptor. Given the limited discriminative ability of EP agonists, we targeted downstream secondary messengers which are specific for each subtype receptors. Accordingly, we used pertussis toxin (300 ng/ml) to block $G\alpha_i$ protein downstream EP3, cAMP analogue (10 μM) to mimic the EP2 and EP4 activation, and A23187, a calcium ionophore (50 ng/ml), to mimic the EP1/3 activation. Although the cAMP analogue or calcium ionophore was unable to produce FGFR1 phosphorylation, pertussis toxin suppressed PGE_2 phosphorylating ability, indicating that EP3 receptor is the predominant subtype involved in PGE_2-induced phosphorylation of FGFR-1. Transfection of all EP receptor subtypes in CHO-FGFR-1 resulted in EPs overexpression but did not allow monitoring of FGFR-1 transactivation in response to PGE_2 for the absence of FGF-2 expression in this cell model (see supplemental Figs. S1 and S2). Thus, to address more specifically the role of EP3 in FGFR-1 activation in response to PGE_2, human umbilical venular endothelial cells were selected, since this cell model has the capability of expressing c-Src and FGF-2 but does not express the EP3 receptor (32). Stimulation of human umbilical venular endothelial cells with 100 nM PGE_2 did not affect FGFR-1 phosphorylation (Fig. 6*C*), demonstrating that the EP3 receptor is responsible for FGFR-1 phosphorylation in endothelium.

Phosphorylation of ERK1/2 by PGE2—We next examined the effect of PGE_2 on ERK1/2 activity, a kinase representing the terminal molecule of the membrane to nucleus signaling elicited by growth factors such as FGF-2; hence, being closely associated with functional events in endothelial cells. PGE_2 promoted ERK1/2 phosphorylation as early as 5 min after its application to endothelial cells (both from bovine and human origin), reaching maximal stimulation (2-fold increase over basal) after 15 min of incubation. PGE_2-induced phosphorylation of ERK1/2 was sensitive to inhibitors of signals described above such as SU5402 (FGFR-1) or PP1 (c-Src) (Fig. 7, *A* and *B*), establishing that both c-Src and FGFR-1 lie upstream to ERK1/2 phosphorylation. Conversely, the VEGFR-2 inhibitor, SU5614, exhibited no effect on ERK1/2 activation (Fig. 7*C*). Note also the detection of intense ERK1/2 phosphorylation in HMVEC-C (Fig. 7*D*) in response to PGE_2 and its dependence on FGFR-1, as shown by its reduced phosphorylation after receptor blockade, indicating the existence of a FGFR-1 mechanism in endothelial cells belonging to a diverse lineage and of human origin.

Furthermore, incubation of EC with inhibitors of the signaling cascade delineated above, such as GM6001 (MMPs), PP1 (c-Src), or U0126 (ERK1/2), severely reduced their ability to migrate in response to PGE_2 (not shown), indicating the functional relevance of the c-Src/MMP/FGFR-1 signaling pathway for the expression of prostanoid activity.

DISCUSSION

This study describes the signaling pathway involved in the action of PGE_2 as a pro-angiogenic molecule in the vascular endothelium. We demonstrated that PGE_2, through the rapid activation and phosphorylation of the tyrosine kinase receptor for the fibroblast growth factor-2, FGFR-1, induces endothelial

cell migration and ERK1/2 activation *in vitro* and pseudocapillary sprouting from aortic rings in *ex vivo* studies.

The work has been performed in cultured endothelial cells, except for few experiments conducted in vascular organ explants, because our aim was to investigate PGE_2 action avoiding the interference of other cell types, such as tumor, stromal, or inflammatory cells. The most significant finding of this study concerns the key role of FGFR-1 as mediator of PGE_2 stimulatory effects in endothelial cells and as a promoter of neovascularization. PGE_2 exerted a concentration-related chemoattractant activity toward endothelial cells, which was stringently dependent on FGFR-1 phosphorylation. PGE_2 also promoted FGFR-1 internalization, as revealed by immunohistochemistry and confocal analysis, leading to its enrichment in the perinuclear area, a feature typical of receptor activation through its natural ligand, FGF-2. Further evidence for FGFR-1 activation as a prerequisite for the functional expression of PGE_2 was found in the enhanced pseudocapillary sprouting which was promptly abolished by FGFR-1 blockade with SU5402.

The mechanism by which PGE_2 promotes FGFR-1 phosphorylation revealed an unforeseen complexity. Because PGE_2 failed to interact directly with the growth factor receptor, we thought of an indirect route leading to receptor activation. Analogous mechanisms have been recently described for other systems (*e.g.* the β-adrenergic and endothelin receptors), which like PGE_2, act through G protein-coupled receptors (33, 34). We focused our attention on c-Src and MMPs as possible intermediate signals between PGE_2 and FGFR-1, the rationale being that a similar pattern of intracellular-extracellular events has been demonstrated to be operant for growth factor tyrosine kinase receptor activation by G protein-coupled receptors (6). Indeed, we found c-Src to be strongly stimulated by PGE_2 in endothelial cells, its activation linked to FGFR-1 phosphorylation as its specific blockade (PP1), abrogated receptor activation. FGFR-1 phosphorylation in response to PGE_2 was also inhibited by applying potent MMP blockers, a finding that implicates MMP activation in the FGFR-1 phosphorylation. Additional characterization indicated that MMP-2 is the MMP involved in the effect. These membrane-bound proteases, upon activation by c-Src, possibly through the intermediate phosphorylation of MT-MMP1 (35), induce shedding of growth factors or chemokines from their membrane-embedded storage sites, as exemplified by EGFR ligands that are released from their glycoprotein storage. The experiments with the neutralizing FGF-2 antibody, which abolished the PGE_2-induced phosphorylation of FGFR-1, illustrate the existence of this extracellular mechanism, clearly indicating that activated MMP-2 mobilized FGF-2 from its membrane storage sites and, in turn, the growth factor promotes FGFR-1 phosphorylation. In addition, the observed failure of PGE_2 to displace FGF-2 from heparin-coated beads highlights the specific nature of the intracellular mechanism that operates through a controlled cascade of signals. ERK1/2, the signal nearest nuclear transcription factors activation, was vigorously phosphorylated by PGE_2. This signal lies downstream from the pathway here examined, as its phosphorylation was sensitive to the selective inhibitors SU5402, PP1, and GM6001. The results on PGE_2-mediated actions extend to cells of different lineage and origin, possibly to the vascular endothelium in general, as indicated by the results obtained by HMVEC-C, a cell of human arterial origin. Both EP3 and EP4 receptors have been implicated in angiogenesis (18, 19). Our evidence suggests that EP3 is the PGE_2 receptor subtype that promotes c-Src and MMP2 activation, leading to FGFR-1 phosphorylation and its subsequent internalization. This cascade of events triggers the angiogenic drive in endothelial cells.

The lack of VEGF involvement in the pro-angiogenic action of PGE_2 in cultured endothelial cells represents an interesting finding. Clearly, the angiogenic pathway of PGE_2, which for the concentrations used in this study pertains to the inflammatory process, and that of VEGF have distinct signaling patterns. In particular, PGE_2 selectively promotes angiogenesis through an endogenous signal transduction pathway, FGF-2/FGFR-1, which operates in an autocrine/paracrine manner to control vascular proliferation. On the contrary, the VEGF/VEGFR-2 system operates in a paracrine manner as its activation is independent of VEGF release from stromal components. However, both pathways share c-Src as a common requirement, which for VEGF is limited to its vascular permeability effects (36). Conceivably, c-Src might be an appropriate target for pharmacological interventions aimed at reducing pathological angiogenesis caused by either inflammation or excess output of VEGF as it occurs in several tumors. In this context the current strategy of targeting single mediators (*e.g.* VEGF with antibodies) has recorded the emergence of resistance or escape from these interventions, reinforcing the concept that multiple independent pathways contribute to pathological angiogenesis, particularly in malignancy often characterized by the coexistence of inflammation and tumor progression. In conclusion, PGE_2 exerts its pro-angiogenic action by recruiting the paracrine-autocrine mechanism characteristic of endothelial cells, *i.e.* stimulation of FGFR-1 through endogenous FGF-2, which supports vascular remodeling.

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