Vascular Endothelial Growth Factor (VEGF)-A165-induced Prostacyclin Synthesis Requires the Activation of VEGF Receptor-1 and -2 Heterodimer*

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We previously reported that vascular endothelial growth factor (VEGF)-A165 inflammatory effect is mediated by acute platelet-activating factor synthesis from endothelial cells upon the activation of VEGF receptor-2 (VEGFR-2) and its coreceptor, neuropilin-1 (NRP-1). In addition, VEGF-A165 promotes the release of other endothelial mediators including nitric oxide and prostacyclin (PGI2). However, it is unknown whether VEGF-A165 is mediating PGI2 synthesis through VEGFR receptor-1 (VEGFR-1) and/or VEGF receptor-2 (VEGFR-2) activation and whether the coreceptor NRP-1 potentiates VEGF-A165 activity. In this study, PGI2 synthesis in bovine aortic endothelial cells (BAEC) was assessed by quantifying its stable metabolite (6-keto prostaglandin F1a, 6-keto PGF1α) by enzyme-linked immunosorbent assay. Treatment of BAEC with VEGF analogs, VEGF-A165 (VEGFR-1, VEGFR-2 and NRP-1 agonist) and VEGF-A121 (VEGFR-1 and VEGFR-2 agonist) (up to 10−9 M), increased PGI2 synthesis by 70- and 40-fold within 15 min. Treatment with VEGFR-1 (placental growth factor and VEGF-B) or VEGFR-2 (VEGFC) agonist did not increase PGI2 synthesis. The combination of VEGFR-1 and VEGFR-2 agonists did not increase PGI2 release. Pretreatment with a VEGFR-2 inhibitor abrogated PGI2 release mediated by VEGF-A165 and VEGF-A121, and pretreatment of BAEC with antisense oligomers targeting VEGFR-1 or VEGFR-2 mRNA reduced PGI2 synthesis mediated by VEGF-A165 and VEGF-A121 up to 79%. In summary, our data demonstrate that the activation of VEGFR-1 and VEGFR-2 heterodimer (VEGFR-1/2-R) is essential for PGI2 synthesis mediated by VEGF-A165 and VEGF-A121, which cannot be reproduced by the parallel activation of VEGFR-1 and VEGFR-2 homodimers with corresponding agonists. In addition, the binding of VEGF-A165 to NRP-1 potentiates its capacity to promote PGI2 synthesis.

Vascular endothelial growth factor (VEGF)1, type A is known as an inflammatory cytokine participating in the wound healing, tissue regeneration, and physiological angiogenesis and also for its capacity to promote pathological angiogenesis in tumor growth, atherosclerosis, and proliferative retinopathies (1, 2). There are five different VEGF-A isoforms of 206, 189, 165, 145, and 121 amino acids and also several VEGF analogs such as placental growth factors 1 and 2 (PIGF-1 and -2), VEGF-B, VEGF-C, VEGF-D, and a viral homolog, VEGF-E (2). The actions of VEGF family members are mediated by the activation of selective tyrosine kinase receptors including VEGF-R1 (Flt-1) and VEGF-R2 (Flk-1/KDR), which are almost exclusively expressed on endothelial cells (ECs) and VEGF-3 (Flt-4), which is mainly limited to the lymphatic endothelium (2). VEGF-A binds to VEGF-R1 and VEGF-R2. PIGF-1, PIGF-2, and VEGF-B bind to VEGF-R1. VEGF-C and VEGF-D bind to VEGF-R2 and VEGF-3, whereas VEGF-E interacts only with VEGF-2 (2, 3). Recent studies also reported that neuropilin-1 (NRP-1), a transmembrane receptor, acts as a coreceptor by enhancing the binding of VEGF-A165 to VEGF-R2 and potentiates various VEGF-A165 biological activities (3–6). Such selectivity is attributable to the presence of VEGF-A exon 7 in VEGF-A165, a domain that is lacking in VEGF-A121, VEGF-C, VEGF-D, and PIGF-1 (4, 7).

Stimulation of ECs with VEGF-A165 can promote prostacyclin (PGI2) synthesis, which is a potent vasodilator and an inhibitor of platelet aggregation (8–10). Consequently, the imbalance in PGI2 production can be involved in the pathophysiology of many thrombotic and cardiovascular disorders. The induction of PGI2 can be mediated upon the activation of different phospholipase A2 enzymes that catalyze the cleavage of arachidonic acid from membrane glycerophospholipids. Subsequently, arachidonic acid is converted in PGI2 by the action of two cyclooxygenase (COX) isoforms, either the constitutive form, COX-1, or the inducible form, COX-2. The newly formed PGI2 then is transformed into PGI2 by the action of the PGI2 synthase (11–14). However, it is unknown whether the members of the VEGF superfamily are mediating PGI2 synthesis either through VEGFR-1 and/or VEGFR-2 activation and whether NRP-1 is contributing to potentiate VEGF-A165-mediated PGI2 synthesis.

During last few years, we have shown that VEGF-A165 increases vascular permeability, endothelial P-selectin translo-
cation, and neutrophil adhesion upon the synthesis of platelet-activating factor (PAF) by ECs (5, 15). We subsequently investigated the contribution of VEGF receptors and assessed that all of these biological activities are mediated through the activation of VEGFR-2 and that these effects are potentiated by VEGFR-1 and VEGFR-2 (4, 5, 16). Consequently, by using VEGF analogs and by regulating VEGF receptor activity, either with selective inhibitors or by antisense treatment, we investigated the contribution of VEGF members and their corresponding receptors on their capacity to promote endothelial PG\(_2\) synthesis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Endothelial cells were harvested from bovine aortas (BAEC) and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 5% fetal bovine serum (Medicorp, Inc., Montreal, Quebec, Canada) and antibiotics (Sigma), and BAEC were characterized as described previously (16) and used between passages 3 and 5.

**Endothelial PG\(_2\) Synthesis—**BAEC were seeded in 6-well plates and cultured up to 3 days post-confluence. Culture medium was removed, and cells were rinsed with HBSS (Hank’s balanced salt solution) plus CaCl\(_2\) (5 mM) with phosphate-buffered saline (PBS) solution or VEGF analogs, VEGF-A\(_{165}\) (PeproTech Inc., Rocky Hill, NJ), VEGF-A\(_{121}\), PGF, VEGF-B, and VEGF-C (R & D Systems, Minneapolis, MN), at various concentrations (10\(^{-11}-10^{-8}\) M) and up to 30 min. In another set of experiments, BAEC were pretreated with selective inhibitors of VEGFR-1 and VEGFR-2 (VTK, VEGF-2 (SU1498), p38 MAPK (SB203580), MEK (PD98059), cytosolic phospholipase A\(_2\) (cPLA\(_2\)) (AACOCF\(_3\)), secreted phospholipase A\(_2\) (sPLA\(_2\)) (scalaradial) (Calbiochem), COX-1 and COX-2 (indomethacin), or PG\(_L\) synthase (tranylcypromine) (Sigma) 15 min prior to stimulation with VEGF-A isoforms. Upon stimulation, the supernatant was collected and PG\(_2\) synthesis was assessed by quantifying its stable metabolite (6-keto PG\(_{1\alpha}\)) according to manufacturer’s instructions (Cayman Chemicals, Ann Arbor, MI).

**Antisense Oligonucleotide Therapy—**We also used an antisense oligonucleotide therapy approach to discriminate the contribution of VEGFR-1 and VEGFR-2 on PGI\(_2\) synthesis mediated by VEGF-A isoforms. BAEC were treated with antisense oligonucleotide sequences complementary to bovine VEGF-1 or VEGF-2 mRNA (GenBank\(^\circ\) accession numbers X94260 and X94268). Antisense oligonucleotide phosphorothioate backbone sequences targeting bovine VEGF-A\(_{165}\) mRNA (AS-R1: 5’-CAAGTGTTTCTACGGAG-3’) and VEGF-2 mRNA (AS-R2: 5’-GCTGCTGTTATTGGTGG-3’), or a scrambled phosphorothioate sequence (AS-Scr: 5’-TGCTGTTATTGGTGGTT3’) (AlphaDNA, Montreal, Quebec, Canada) were used. The antisense oligomers were chosen based on their capacity to selectively abrogate the protein expression of the genes targeted, as described previously (16). BAEC were seeded at 5 × 10\(^5\) cells/well in 6-well plates in Dulbecco’s modified Eagle’s medium, 5% fetal bovine serum, and antibiotics (10 M) up to 3 days post-confluence. Culture medium was removed, cells were rinsed and stimulated in HBSS/HEPES + CaCl\(_2\) (5 mM) with PBS or VEGF-A isoforms, and PG\(_L\) synthesis was quantified as described above.

**Preparation of Glutathione S-Transferase (GST)-VEGF-A\(_{165}\) Exon 7 Fusion Protein—**To evaluate the possible potentiating effect of NRP-1 on VEGF-A\(_{165}\)-induced PG\(_L\) synthesis, we produced a GST fusion protein encoding exon 7 of human VEGF-A\(_{165}\) (GST-Ex7). The construct of GST fusion protein-exon 7 was generously provided by Dr. Shary Soker, Wake Forest University, Winston-Salem, NC. Escherichia coli (DH5\(_a\)) were transformed with pGEX-2TK or pGEX-exon 7 vectors to produce GST and GST-Ex7 proteins. The recombinant proteins were purified from bacterial lysates using glutathione and heparin affinity chromatographies as described previously (5, 6).

**Western Blot Analyses of VEGF Receptors Expression and Phosphorylation—**BAEC were cultured up to 3 days post-confluence, and cells were rinsed, incubated on ice, and stimulated in HBSS/HEPES + CaCl\(_2\) (5 mM) with 1 mg/ml bovine serum albumin. In some experiments, BAEC were pretreated with PBS or VEGF receptor inhibitors (SU1498 or VTK) 15 min prior to the addition of VEGF-A\(_{165}\) or VEGF-A\(_{121}\) and the cells were kept on ice for an additional 15 min. The cells then were stimulated for 7.5 min at 37 °C and placed again on ice. In another set of experiments, BAEC were stimulated as above with VEGF analogs only. Upon stimulation, the medium was removed, cells were washed, and lysates were prepared. Western blot analyses were performed as described previously (4, 5, 16). The primary antibodies used were mouse monoclonal anti-human VEGF-1 (clone Flt-11, Sigma), polyclonal rabbit anti-mouse VEGF-2, and goat anti-human NRP-1 IgG antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA). Membranes were stripped using Re-Blot Plus Strong stripping solution (Chemicon International, Temecula, CA) for 20 min and reprobed with a mouse monoclonal anti-phosphoryroline IgG (clone 4G10, 1:4000 dilution, Upstate Biotechnology Inc., Lake Placid, NY) to determine VEGF-1 and VEGF-2 phosphorylation. Kaleidoscope molecular weight markers (Bio-Rad) were used as molecular mass standards for SDS-PAGE immunoblotting experiments. Immunoreactive bands were visualized by ECL, digitized using a two-dimensional gel scanner, and quantified using Quantity One software (Bio-Rad).

In another set of experiments, we assessed by Western blot analyses the expression and activation of selective enzymes involved in the cell signaling pathway leading to PG\(_L\) synthesis. Studies were performed as described above. Primary antibodies used for immunoprecipitations and Western blot analyses were as follows: rabbit polyclonal anti-human phospho-p42/p44 MAPK and anti-human phospho-cPLA\(_2\) (Cell Signaling Technology Inc., Beverly, MA) and mouse monoclonal anti-ovine COX-1 and mouse monoclonal anti-human COX-2 (Cayman Chemicals) IgGs. The membranes then were stripped and reprobed with rabbit anti-rat p42/p44 MAPK (Cell Signaling) and mouse monoclonal anti-ovine PLA\(_2\) (Cayman Chemicals) IgGs.

**Western Blot Analysis of PG\(_L\) Synthase S-Nitrosylation, and Cyclooxygenases-1 and -2—**BAEC were cultured up to 3 days post-confluence and treated prior to stimulation as described above. Cells were incubated on ice for 30 min with either VEGF-A\(_{165}\) or VEGF-C (10\(^{-9}\) M) and then stimulated for 5–15 min at 37 °C to return to ice. Upon stimulation, the medium was removed, cells were washed, and lysates were prepared. Immunoreactive bands were visualized with a rabbit polyclonal anti-bovine PG\(_L\) synthase IgG (Cayman Chemicals). Samples were separated on a 10% SDS-PAGE, and Western blot analyses were performed as described previously (4, 5, 16). A mouse monoclonal anti-nitrotyrosine IgG (Cayman Chemicals) was used to assay the S-nitrosylation level of PG\(_L\) synthase. The membranes then were stripped and reprobed with rabbit polyclonal anti-bovine PG\(_L\) synthase IgG as described above.

**Statistical Analysis—**Data are presented as the mean ± S.E. Statistical comparisons were made by analysis of variance followed by a Bonferroni’s t test for multiple comparisons. Differences were considered significant when p < 0.05.

**RESULTS**

Effect of VEGF Analogs and Corresponding Receptors on Prostacyclin Synthesis—PG\(_L\) synthase in post-confluent BAEC was quantified by measuring its stable metabolite 6-keto PG\(_{1\alpha}\) by ELISA. First, we performed a time-dependent (5–30 min) and concentration-dependent (10\(^{-11}-10^{-8}\) M) assay to assess how VEGF-A\(_{165}\) mediates PG\(_L\) synthesis. In control PBS-treated cells, the production of 6-keto PG\(_{1\alpha}\) did not change in function of time. At 15 min post-treatment, the concentration of 6-keto PG\(_{1\alpha}\) was 0.657 ± 0.056 ng/10\(^6\) cells. Treatment with VEGF-A\(_{165}\) (10\(^{-9}\) M) induced a rapid and transient PG\(_L\) synthesis. Within 5 min, we observed a 48-fold increase that reached a plateau within 10 min (70-fold increase). In addition, VEGF-A\(_{165}\) (10\(^{-11}-10^{-8}\) M) increased PG\(_L\) synthesis by 5-, 30-, and 70-fold, respectively. Interestingly, at the highest concentration (10\(^{-8}\) M), VEGF-A\(_{165}\) was less efficient (30-fold increase) in mediating PG\(_L\) synthesis (Fig. 1A). B and C illustrate that to determine which VEGF receptors are involved in PG\(_L\) synthesis, we used selective VEGF analogs. Treatment with VEGF-A\(_{165}\) isoform (10\(^{-11}-10^{-8}\) M), which like VEGF-A\(_{165}\) binds to VEGFR-1 and VEGFR-2 but not to NRP-1 coreceptor, induced a significant but reduced PG\(_L\) synthesis (40-fold increase at 10\(^{-9}\) M) compared with VEGF-A\(_{165}\) (Fig. 1C). These latter data suggest that NRP-1 coreceptor might contribute to potentiate VEGF-A\(_{165}\) capacity to promote PG\(_L\) synthesis. To support this hypothesis, we treated BAEC with PBS, VEGF-A\(_{165}\), and VEGF-A\(_{121}\) and confirmed by Western blot analysis the capacity of VEGF-A\(_{165}\) as opposed to VEGF-A\(_{121}\)–PBS-treated cells to promote VEGF-2-NRP-1 complex formation (Fig. 2A). BAEC then were pretreated with GST-Ex7 of human.
VEGF-A165 to block the interaction of VEGF-A165 with NRP-1. Exon 7 encodes a domain not present in VEGF-A121 that is responsible for the binding of VEGF-A165 to NRP-1 (3, 5). Pretreatment of BAEC with GST-Ex7 (up to 10⁻⁷ M) 15 min prior to stimulation with VEGF-A165 (10⁻⁹ M) reduced PGI₂ synthesis to the level induced by VEGF-A121 (Fig. 2B). Pretreatment with GST (up to 10⁻⁷ M), without the exon 7 insert did not alter VEGF-A165-induced PGI₂ synthesis, and neither GST-Ex7 nor GST (up to 10⁻⁷ M) altered significantly the basal level of PGI₂ synthesis or VEGF-A121-induced PGI₂ synthesis (Fig. 2B).

Because VEGF-A165 and VEGF-A121 are both capable of activating VEGFR-1 and VEGFR-2, we treated BAEC with selective analogs for VEGFR-1 (PlGF and VEGF-B) and VEGFR-2 (VEGF-C) to verify the contribution of each receptor under their homodimeric conformations on PGI₂ synthesis. Treatment with PlGF, VEGF-B, or VEGF-C (10⁻¹¹-10⁻⁸ M) for 15 min did not promote the release of PGI₂ (Fig. 3, A–C). To assess whether a parallel activation of VEGFR-1 and VEGFR-2 homodimers may induce PGI₂ release, BAEC were treated with the combination of VEGFR-1 analogs PlGF or VEGF-B with VEGFR-2 analog VEGF-C at 10⁻⁹ M and for 15 min. Such a combination did not increase PGI₂ synthesis (Fig. 3D).

Phosphorylation of VEGF Receptors by Corresponding VEGF Analogs—Because VEGF-A165 and VEGF-A121 were the only VEGF analogs capable of mediating endothelial PGI₂ synthesis, we performed Western blot analyses to confirm the expression of VEGFR-1 and VEGFR-2 and the capacity of VEGF analogs to activate them. BAEC were treated with VEGF analogs for 7.5 min, which is the suitable time to detect the phosphorylation of VEGF receptors as described previously (16). Cell lysates were immunoprecipitated either with anti-VEGFR-1 or anti-VEGFR-2 IgGs. VEGF-1 and VEGF-2 protein expression in BAEC was detected by immunoblotting, which is in agreement with previous reports (Fig. 4, A and B, upper bands) (17). The membranes were stripped, and the detection of VEGFR-1 and VEGFR-2 phosphorylation was performed by reprobing the membranes with anti-phosphotyrosine IgG. Treatment with VEGF-A165 and VEGF-A121 (10⁻⁹ M) increased the phosphorylation of VEGFR-1 by 10.6- and 7.3-fold as compared with PBS-treated cells, whereas equivalent treat-
ment with PlGF or VEGF-B did not increase VEGFR-1 phosphor-
ylation (Fig. 4A, lower panel). Although we could not de-
tect the phosphorylation of VEGFR-1-mediated by PlGF or
VEGF-B, they were not deprived of biological activities since
they were capable of promoting endothelial P-selectin translo-
cation (data not shown and as described previously) (5). Next,
we assessed the capacity of VEGF-A165, VEGF-A121, and
VEGF-C (10^{-9} M) to mediate VEGFR-2 phosphorylation. Such
treatment increased the phosphorylation of VEGFR-2 by 57.8-,
24.7-, and 5.8-fold compared with PBS-treated cells (Fig. 4B,
lower panel).

**PGL_2 Synthesis Requires VEGFR-1 and VEGFR-2 Het-
erodimerization—**Our data demonstrate that the activation of
VEGFR-1 or VEGFR-2 homodimers alone or in parallel with
selective VEGFR-1 or VEGFR-2 analogs did not promote PGL_2
synthesis. Consequently, we speculated that VEGF-A isoforms
induce PGI2 synthesis through the activation of VEGFR-1/R-2 heterodimer. By Western blot analysis, we observed in PBS-treated cells that VEGFR-1 and VEGFR-2 subunits can constitutively be present under heterodimeric VEGFR-1/R-2 state and that one treatment either with VEGF-A165 or VEGF-C did not modulate VEGFR-1/R-2 dimerization (Fig. 5). Next, to demonstrate that PGI2 synthesis mediated by VEGF-A165 and VEGF-A121 is driven through the activation of VEGFR-1/R-2 heterodimer, BAEC were treated with selective antisense oligomers targeting VEGFR-1 or VEGFR-2 mRNA. We showed previously that such an approach at the concentration used (5 \times 10^{-7} \text{ M/daily}) abrogated selectively the protein expression of VEGFR-1 or VEGFR-2 by over 90% and the biological activities investigated by 80–100% (16). Treatment with selective antisense oligomers targeting VEGFR-1 (AS-R1) or VEGFR-2 (AS-R2) mRNA reduced, by 79 and 71%, the synthesis of PGI2 mediated by VEGF-A165 and, by 73 and 62%, the synthesis of PGI2 mediated by VEGF-A121, respectively (Fig. 6A). As negative control, BAEC were treated with a scrambled oligomer sequence, which did not decrease significantly the level of PGI2 synthesis mediated by VEGF-A165 and VEGF-A121. In addition, the treatment of BAEC with antisense or scrambled oligomers did not affect the basal level of PGI2 synthesis in PBS-treated cells (Fig. 6A).

To support the latter study, we used as well a pharmacological approach. Pretreatment of ECs with a selective VEGFR-1 and VEGFR-2 inhibitor (VTK, 10^{-5} \text{ M}; IC_{50} = 2.0 and 0.1 \times 10^{-8} \text{ M}, respectively) (18) abrogated by 100 and 90% the synthesis of PGI2 mediated by VEGF-A165 and VEGF-A121, respectively. Similarly, the blockade of VEGFR-2 activity with SU1498 (selective VEGFR-2 inhibitor; 5 \times 10^{-6} \text{ M}; IC_{50} = 7 \times 10^{-8} \text{ M}) (5, 19) was sufficient as well to abrogate by 96 and 95% the synthesis of PGI2 mediated by VEGF-A165 and VEGF-A121, respectively (Fig. 6B).

To verify the selectivity of VEGF receptor inhibitors, we assessed their corresponding inhibitory effect on VEGFR-1 and VEGFR-2 phosphorylation mediated by VEGF-A165. Pretreatment of BAEC with SU1498 (5 \times 10^{-6} \text{ M}) 15 min prior to stimulation with VEGF-A165 (10^{-7} \text{ M}, 7.5 min) did not affect the phosphorylation of VEGFR-1 but prevented the phosphorylation of VEGFR-2. Pretreatment with VTK prevented the phosphorylation of VEGFR-1 and VEGFR-2 mediated by VEGF-A165 (Fig. 7) by 100 and 83%.

**Cell Signaling Pathways by Which VEGF-A165 Induces PGI2 Synthesis**—In previous studies, we have shown that VEGF-A165 induces PAF synthesis upon the activation of the VEGFR-2/NRP-1 complex and requires the activation of both p38 and p42/44 MAPKs and subsequent activation of sPLA2 type V (sPLA2-V) (20, 21). Because VEGF-A165 induces PGI2 synthesis upon VEGFR-1/2 activation, which is also potentiated by NRP-1 coexpression, we wanted to assess the similarities in the cell signaling pathways involved in PAF and PGI2 synthesis. Pretreatment of BAEC with p38 MAPK inhibitor SB203580 (10^{-5} \text{ M}; IC_{50} = 6 \times 10^{-7} \text{ M}) (22) did not prevent PGI2 synthesis mediated by VEGF-A165 (10^{-5} \text{ M}). However, pretreatment with MEK inhibitor PD98059 (10^{-5} \text{ M}; IC_{50} = 10^{-6} \text{ M}) (23), which prevents p42/44 MAPK phosphorylation, abrogated the synthesis of PGI2 mediated by VEGF-A165 (Fig. 8). BAEC then were pretreated with PLA2 inhibitors. Pretreatment with a broad range inhibitor of sPLA2, scalaradial (10^{-5} \text{ M}) (21), which is capable of preventing VEGF-A165-mediated PAF synthesis, did not reduce the release of PGI2 significantly, whereas a pretreatment with cPLA2 inhibitor AOCOCF3 (5 \times 10^{-5} \text{ M}, IC_{50} = 10^{-7} \text{ M}) (24) inhibited the release of PGI2 mediated by VEGF-A165 (Fig. 8) by 86%. Finally, a pretreatment of BAEC, either with a non-selective inhibitor of COX-1 and COX-2 (indomethacin; 10^{-5} \text{ M}, IC_{50} = 0.1 and 6 \times 10^{-6} \text{ M}, respectively) (25) or with a selective inhibitor of PGI2 synthase (tranylcypromine; 3 \times 10^{-3} \text{ M}) (26), abrogated completely the synthesis of PGI2 mediated by VEGF-A165 (Fig. 8). To assess whether inducible COX-2 might be present and contribute to constitutive COX-1 activity, we performed Western blot analyses and detected only the protein expression of COX-1 (data not shown).

**Dentrosylation of Prostacyclin Synthase Is Required to Promote PGI2**—Under quiescent state, PGI2 synthase is S-nitrosylated, thus preventing its capacity to convert its substrate, PGH2, into PGI2 (27). In a previous study, we have shown that VEGF-A165 and VEGF-C are capable of mediating PAF synthesis upon VEGF-2 homodimer activation (4, 16). In the current study, we also observed that VEGF-A165 and VEGF-C are both capable to promote the phosphorylation p42/44 MAPK and cPLA2 (data not shown), which are essential to promote arachidonic acid release (8, 28). However, VEGF-C as opposed to VEGF-A165 cannot induce PGI2 synthesis. Consequently, we hypothesized that VEGF-C may not be capable of promoting the S-denitrosylation of PGI2 synthase, which is required for PGI2 production (27, 29).

Thus, we assessed, by Western blot analysis, the capacity of VEGF-A165 and VEGF-C to regulate the level of PGI2 synthase nitrosylation. Treatment with VEGF-A165 (10^{-8} \text{ M}) induced a rapid/immediate S-denitrosylation of PGI2 synthase by 30%, which returned to its basal nitrosylated state after 15 min of treatment. At the opposite, treatment with VEGF-C (10^{-5} \text{ M}) had no such effect (Fig. 9).

**DISCUSSION**

Previous studies reported the capacity of VEGF-A165 to promote PGI2 synthesis. However, there were no data defining the contribution of VEGF receptor(s) (8, 30, 31). In the current study, we observed that only VEGF-A isoforms (VEGF-A165 and VEGF-A121) were able to promote an acute endothelial PGI2 synthesis, whereas the stimulation of BAEC with selective VEGFR-1 (PIGF or VEGF-B) or VEGFR-2 (VEGF-C) agonists, alone or combined, had no such effect. In addition, VEGF-A165 was approximately twice potent as VEGF-A121 to promote PGI2 synthesis. Pretreatment of ECs with a GST-Ex7 fusion protein, which prevents the binding of VEGF-A165 to NRP-1, reduced the capacity of VEGF-A165 to promote PGI2 synthesis to the level mediated by VEGF-A121, which does not bind to NRP-1. Together, these data suggest that the activation of VEGFR-1 and VEGFR-2 homodimers by their corresponding analogs is not sufficient to promote PGI2 synthesis and depends on the activation of the VEGFR-1/R-2 heterodimer. Furthermore, NRP-1 potentiates the capacity of VEGF-A165 to promote PGI2 synthesis.

To assess the requirement of VEGFR-1/R-2 activation for promoting PGI2 synthesis mediated by VEGF-A isoforms, we used antisense gene therapy and pharmacological approaches to regulate the expression and the activation of VEGF receptors. In a first series of experiments, BAEC were treated with antisense oligomers targeting selectively VEGFR-1 or
VEGFR-2 mRNA that we previously had defined for their capacity to down-regulate selectively their corresponding protein expression by over 90% and related biological activities (16). Such treatment with antisense oligomers for VEGFR-1 or VEGFR-2 mRNA abrogated by over 70% the synthesis of PGI2 mediated by VEGF-A165 and VEGF-A121 (Fig. 6). In another set of experiments, BAEC were pretreated with selective VEGF receptor inhibitors. Pretreatment of BAEC with a VEGFR-2 inhibitor, SU1498, prevented VEGF-A activation by VEGF-A isoforms and PGI2 synthesis. Together, these data demonstrate that the activation of both receptors under heterodimeric state (VEGFR-1/R-2) is required for PGI2 synthesis. Indeed, it cannot be due either to an independent or parallel activation of VEGFR-1 and/or VEGFR-2 homodimers by VEGF-A isoforms.

Figure 6. Contribution of VEGF receptors on PGI2 synthesis induced by VEGF-A isoforms. A. BAEC were plated at 5 × 10^6 cells/well in 6-well plates. Antisense oligomers targeting VEGFR-1 (AS-R1) or VEGFR-2 (AS-R2) mRNA or a scrambled oligomer (As-Scr) were added daily (5 × 10^{-7} M) up to 3 days post-confluence. Cells were stimulated with PBS, VEGF-A_{165}, or VEGF-A_{121} (10^{-9} M) for 15 min. B. Post-confluent BAEC were pretreated with a VEGFR-1 and VEGFR-2 tyrosine kinase inhibitor, VTK (10^{-5} M), or a VEGFR-2 inhibitor, SU1498 (5 × 10^{-6} M) 15 min prior to stimulation with PBS, VEGF-A_{165}, or VEGF-A_{121} (10^{-9} M) for 15 min. PGI2 synthesis was assessed by quantifying its stable metabolite (6-keto PGF_{1α}) by ELISA. ***, p < 0.001 versus PBS-treated cells; §§, p < 0.01, and §§§, p < 0.001 versus cells treated with VEGF-A isoforms.
because the blockade of VEGFR-1 or VEGFR-2 expression and corresponding activation were sufficient to prevent PGI2 synthesis. Furthermore, parallel activation of VEGFR-1/R-1 and VEGFR-2/R-2 by selective agonists did not promote PGI2 synthesis.

Although we detected the phosphorylation of VEGFR-1 mediated by VEGF-A isoforms, we could not detect its phosphorylation upon a treatment with PI GF and VEGF-B. Consequently, one might argue that this could explain why PI GF and VEGF-B were unable to promote PGI2 synthesis. However, this hypothesis does not stand because we observed in a different study that PI GF and VEGF-B at the same concentrations and within the same time period were both capable to promote a significant increase of endothelial P-selectin translocation, despite our incapacity to detect VEGFR-1 phosphorylation.

Our data are also in agreement with previous reports that have shown that PI GF and VEGF-B were capable of promoting specific biological activities, namely on endothelial and monocyte tissue factor production and migration of monocytes, despite the fact that VEGFR-1 phosphorylation was undetectable (32–36). This could also be explained by the fact that the tyrosine kinase activity of VEGFR-1 is one order of magnitude lower than that of VEGFR-2 and that, in function of the ligands used, VEGFR-1 can autophosphorylate differently, rendering difficult the detection of its autophosphorylation (37, 38).

The lack of PGI2 synthesis upon stimulation with VEGF-C also was not due to its incapacity to activate VEGFR-2, because VEGF-C was capable of promoting VEGFR-2 phosphorylation (Fig. 4) and selective biological activities including endothelial P-selectin translocation, endothelial cell migration and proliferation, and PAF synthesis (4, 5, 16, 39). Together, these observations strengthened our hypothesis that VEGF-A isoforms require the activation of VEGFR-1/R-2 heterodimer to support PGI2 synthesis.

Our observations are also supported by recent studies that have demonstrated that, in native unstimulated and VEGF-A165-treated ECs, VEGFR-1 was consistently detected in anti-VEGFR-2 immunoprecipitates, indicating that both receptors spontaneously form heterodimer (38). Porcine aortic endothelial cells (PAEC) transfected with VEGFR-1 and VEGFR-2 proved as well the heterodimerization of VEGFR-1/R-2. Stimulation of all three PAEC-transfected cell lines expressing VEGFR-1, VEGFR-2, and VEGFR-1/R-2 with VEGF-A165 resulted in signal transduction with different efficiencies and biological activities (40). For instance, migration of PAEC co-expressing VEGFR-1/R-2 toward VEGF-A165 was more efficient than migration of PAEC expressing VEGFR-2 alone, even though similar number of VEGFR-2 subunits were expressed in transfected PAEC (40). These data suggest that the signal transduction properties of VEGFR-2 are affected by its dimerization with VEGFR-1 and its transphosphorylation. This is in agreement with a recent study reporting that VEGF-A165 can induce a strong phosphorylation of VEGFR-1 tyrosine residue Tyr-1213 and to lesser extent Tyr-1242 and Tyr-1333, whereas PI GF induced the phosphorylation of Tyr-1309 but not Tyr-1213 (38). Such differences in the activation of VEGF receptors by various agonists termed “agonist trafficking” might explain the distinct biologic activities of VEGF-A165 and its analogs.

Because VEGF-A165 and VEGF-A121 are the only VEGF analogs capable to bind VEGFR-1/R-2 heterodimer, they might provide an exclusive transphosphorylation of VEGFR-1 and VEGFR-2 subunits that appears to be essential to govern cell signaling leading to PGI2 synthesis.

In previous studies, we have shown that VEGF-A165 induces PAF synthesis upon the activation of VEGFR-2/R-2 homodimer, which is potentiated by the presence of NRP-1 coreceptor (4, 16). Furthermore, the synthesis of PAF requires the activation of both p38 and p42/44 MAPKs and subsequent activation of sPLA2-V (20, 21). Interestingly, the stimulation of VEGFR-1 did not promote PAF synthesis and was not required to support VEGF-A165-mediated PAF synthesis through VEGFR-2 activation (4, 16).

In order to assess the different contribution of VEGF receptors for the induction of PAF and PGL2 synthesis mediated by VEGF-A165, we investigated the cell signaling pathways involved in PGL2 synthesis. Previous studies highlighted the contribution of p42/44 and/or p38 MAPK on PGL2 synthesis (8, 41–45). To discriminate the potential role played by both MAPKs, we used specific inhibitors, namely PD98059, which inhibits MEK, the enzyme upstream and responsible for p42/44 MAPK activation, and SB203580, a specific inhibitor of p38 MAPK activation (20). In our study, the blockade of p42/44
Fig. 9. S-Nitrosylation of PGI2 synthase in the presence of VEGF-A165 and VEGF-C. Post-confluent BAEC were stimulated time-dependently with PBS, VEGF-A165, or VEGF-C (10^{-8} M). Cell lysates (500 μg of total proteins) were immunoprecipitated with PGI2 synthase IgG. Western blot analyses were performed with an anti-nitrotyrosine IgG to assess the S-nitrosylation level of PGI2 synthase, and then the membranes were stripped and reprobed with an anti-PGI2 synthase IgG as described above. The nitrosylation level of PGI2 synthase upon stimulation with VEGF-A165 or VEGF-C was expressed as a function of the protein expression level of PGI2 synthase, and the results were normalized to PBS-treated cells.

In conclusion, we observed for the first time the necessity for VEGF-A isoforms to activate VEGFR-1/R-2 heterodimer, a rapid and transient of PGI2 synthase denitrosylation and therefore PGI2 synthase activity. Because VEGF-C is also capable of promoting VEGFR-2 activation and PAF synthesis but not PGI2 synthesis, we investigated by Western blot analyses its capacity to induce the phosphorylation of p42/44 MAPK and cPLA2. However, because it is well established that p42/44 MAPK can promote the phosphorylation of cPLA2 (8, 46) and that p42/44 MAPK activation is essential for PAF synthesis (20), we assessed the contribution of both PLA2 on PGI2 synthesis. The blockade of cPLA2 prevented completely the synthesis of PGI2, whereas the inhibition of sPLA2 induced a non-significant decrease of PGI2 synthesis. Such a difference between VEGF-C and VEGF-A165 to promote PGI2 synthesis. Thus, the rate-limiting step can be due to a possible difference in their capacity to promote PGI2 synthase activity.

Because VEGF-C is also capable of selecting biological activity in occurring PGI2 synthase. We have also observed that, even if VEGF-C and VEGF-A165 are capable of activating the same signaling pathways leading to endothelial arachidonic acid release, they showed a different capacity to promote PGI2 synthase denitrosylation and therefore PGI2 synthesis. Such a difference would be attributable to the exclusive capacity of VEGF-A isoforms to activate VEGFR-1/R-2 heterodimer.
ERODIMER. These findings provide novel informative data leading to a better comprehension of the selective roles played by VEGF family members and their corresponding receptors on the regulation and maintenance of vascular tone and integrity.

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