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Vascular Endothelial Growth Factor (VEGF)-A_{165}-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)-A_{165} 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-A_{165} promotes the release of other endothelial mediators including nitric oxide and prostacyclin (PGI_2). However, it is unknown whether VEGF-A_{165} is mediating PGI_2 synthesis through VEGF receptor-1 (VEGFR-1) and/or VEGF receptor-2 (VEGFR-2) activation and whether the coreceptor NRP-1 potentiates VEGF-A_{165} activity. In this study, PGI_2 synthesis in bovine aortic endothelial cells (BAEC) was assessed by quantifying its stable metabolite (6-keto prostaglandin $F_{1\alpha}$, 6-keto $PGF_{1\alpha}$) by enzyme-linked immunosorbent assay. Treatment of BAEC with VEGF analogs, VEGF-A_{165} (VEGFR-1, VEGFR-2 and NRP-1 agonist) and VEGF-A_{121} (VEGFR-1 and VEGFR-2 agonist) (up to 10^{-9} M), increased PGI_2 synthesis by 70- and 40-fold within 15 min. Treatment with VEGFR-1 (placental growth factor and VEGF-B) or VEGFR-2 (VEGF-C) agonist did not increase PGI_2 synthesis. The combination of VEGFR-1 and VEGFR-2 agonists did not increase PGI_2 release. Pretreatment with a VEGFR-2 inhibitor abrogated PGI_2 release mediated by VEGF-A_{165} and VEGF-A_{121}, and pretreatment of BAEC with antisense oligomers targeting VEGFR-1 or VEGFR-2 mRNA reduced PGI_2 synthesis mediated by VEGF-A_{165} and VEGF-A_{121} up to 79%. In summary, our data demonstrate that the activation of VEGFR-1 and VEGFR-2 heterodimer (VEGFR-1/R-2) is essential for PGI_2 synthesis mediated by VEGF-A_{165} and VEGF-A_{121}, which cannot be reproduced by the parallel activation of VEGFR-1 and VEGFR-2 homodimers with corresponding agonists. In addition, the binding of VEGF-A_{165} to NRP-1 potentiates its capacity to promote PGI_2 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 (PlGF-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 VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), which are almost exclusively expressed on endothelial cells (ECs) and VEGFR-3 (Flt-4), which is mainly limited to the lymphatic endothelium (2). VEGF-A binds to VEGFR-1 and VEGFR-2. PlGF-1, PlGF-2, and VEGF-B bind to VEGFR-1. VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3, whereas VEGF-E interacts only with VEGFR-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-A_{165} to VEGFR-2 and potentiates various VEGF-A_{165} biological activities (3–6). Such selectivity is attributable to the presence of VEGF-A exon 7 in VEGF-A_{165}, a domain that is lacking in VEGF-A_{121}, VEGF-C, VEGF-D, and PlGF-1 (4, 7).

Stimulation of ECs with VEGF-A_{165} can promote prostacyclin (PGI_2) synthesis, which is a potent vasodilator and an inhibitor of platelet aggregation (8–10). Consequently, the imbalance in PGI_2 production can be involved in the pathophysiology of many thrombotic and cardiovascular disorders. The induction of PGI_2 can be mediated upon the activation of different phospholipase A_2 enzymes that catalyze the cleavage of arachidonic acid from membrane glycerophospholipids. Subsequently, arachidonic acid is converted in PGH_2 by the action of two cyclooxygenase (COX) isoforms, either the constitutive form, COX-1, or the inducible form, COX-2. The newly formed PGH_2 then is transformed into PGI_2 by the action of the PGI_2 synthase (11–14). However, it is unknown whether the members of the VEGF superfamily are mediating PGI_2 synthesis either through VEGFR-1 and/or VEGFR-2 activation and whether NRP-1 is contributing to potentiate VEGF-A_{165}-mediated PGI_2 synthesis.

During last few years, we have shown that VEGF-A_{165} increases vascular permeability, endothelial P-selectin translo-

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; PlGF, placental growth factor; EC, endothelial cell; VEGFR, VEGF receptor; NRP, neuropilin; PGI_2, prostacyclin; COX, cyclooxygenase; BAEC, bovine aortic endothelial cells; ELISA, enzyme-linked immunosorbent assay; PAF, platelet-activating factor; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; $cPLA_2$, cytosolic phospholipase A_2; $sPLA_2$, secreted phospholipase A_2; $SPLA_2$-V, $sPLA_2$, type V; PGH_2, prostaglandin H_2; 6-keto $PGF_{1\alpha}$, 6-keto prostaglandin $F_{1\alpha}$; GST, glutathione *S*-transferase; Ex7, exon 7; PAEC, porcine aortic endothelial cells; HBSS, Hank's balanced salt solution; VTK, VEGFR-1 and VEGFR-2 tyrosine kinase inhibitor.

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 the presence of NRP-1 (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 PGI_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 PGI_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)/HEPES (10 mM, pH 7.4) (Sigma). Cells were stimulated in HBSS/HEPES 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}, PlGF, 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), VEGFR-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 PGI_2 synthase (tranylcypromine) (Sigma) 15 min prior to stimulation with VEGF-A isoforms. Upon stimulation, the supernatant was collected and PGI_2 synthesis was assessed by quantifying its stable metabolite (6-keto $PGF_{1\alpha}$) 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 VEGFR-1 or VEGFR-2 mRNA (GenBank™ accession numbers X94263 and 94298). Antisense oligonucleotide phosphorothioate backbone sequences targeting bovine VEGFR-1 mRNA (AS-R1: 5′-CAA AGA TGG ACT CGG GAG-3′) and VEGFR-2 mRNA (AS-R2: 5′-GCT GCT CTG ATT GTT GGG-3′) or a scrambled phosphorothioate sequence (AS-Scr: 5′-TGC TGG CAT GTG CGT TGT-3′) (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 with or without oligomers (5×10^{-7} M/daily) 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 PGI_2 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 PGI_2 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. Shay Soker, Wake Forest University, Winston-Salem, NC. *Escherichia coli* (DH5α) were transformed with pGEX-2TK or p2TK-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) plus 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 VEGFR-1 (clone Flt-11, Sigma), polyclonal rabbit anti-mouse VEGFR-2, and goat anti-human NRP-1 IgG antibodies (Santa Cruz Biotechnology Inc., 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-phosphotyrosine IgG (clone 4G10, 1:4000 dilution, Upstate Biotechnology Inc., Lake Placid, NY) to determine VEGFR-1 and VEGFR-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 PGI_2 synthesis. Studies were performed as described above. Primary antibodies used for immunoprecipitations and Western blot analyses were as follows: rabbit polyclonal anti-human phospho-p42/44 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 p42/44 MAPK (Cell Signaling) and mouse monoclonal anti-human $cPLA_2$ (Santa Cruz Biotechnology).

Western Blot Analysis of PGI_2 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 and returned to ice. Upon stimulation, the medium was removed, cells were washed, and lysates were prepared. Immunoprecipitation of cell lysate was performed with a rabbit polyclonal anti-bovine PGI_2 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 assess the *S*-nitrosylation level of PGI_2 synthase. The membranes then were stripped and reprobed with rabbit polyclonal anti-bovine PGI_2 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—PGI_2 synthesis in post-confluent BAEC was quantified by measuring its stable metabolite 6-keto $PGF_{1\alpha}$ 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 PGI_2 synthesis. In control PBS-treated cells, the production of 6-keto $PGF_{1\alpha}$ did not change in function of time. At 15 min post-treatment, the concentration of 6-keto $PGF_{1\alpha}$ was 0.657 ± 0.056 ng/10^6 cells. Treatment with VEGF-A_{165} (10^{-9} M) induced a rapid and transient PGI_2 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^{-9} M) increased PGI_2 synthesis by 5-, 30-, and 70-fold, respectively. Interestingly, at the highest concentration (10^{-8} M), VEGF-A_{165} was less efficient (33-fold increase) in mediating PGI_2 synthesis (Fig. 1, *A* and *B*).

To determine which VEGF receptors are involved in PGI_2 synthesis, we used selective VEGF analogs. Treatment with VEGF-A_{121} 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 PGI_2 synthesis (40-fold increase at 10^{-9} M) compared with VEGF-A_{165} (Fig. 1*C*). These latter data suggest that NRP-1 coreceptor might contribute to potentiate VEGF-A_{165} capacity to promote PGI_2 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}- or PBS-treated cells to promote VEGFR-2·NRP-1 complex formation (Fig. 2*A*). BAEC then were pretreated with GST-Ex7 of human

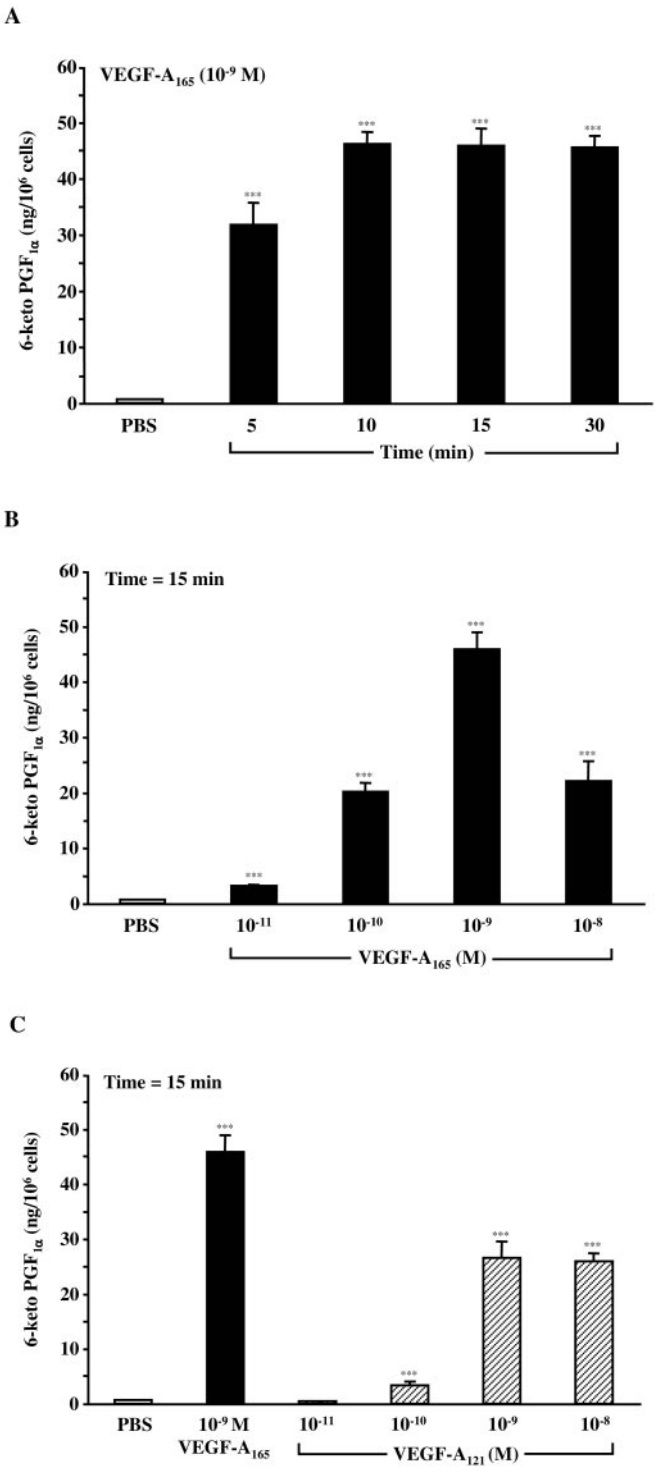

FIG. 1. **PGI_2 synthesis mediated by VEGF-A isoforms.** Post-confluent BAEC were stimulated either with control PBS solution or with VEGF-A_{165} (10^{-9} M) up to 30 min (*A*) or concentration-dependently (10^{-11}-10^{-8} M) with VEGF-A_{165} (*B*) or VEGF-A_{121} (*C*) for 15 min. Synthesized PGI_2 was assessed by quantifying its stable metabolite 6-keto $PGF_{1\alpha}$ by ELISA. ***, $p < 0.001$ *versus* PBS-treated cells.

VEGF-A_{165} to block the interaction of VEGF-A_{165} with NRP-1. Exon 7 encodes a domain not present in VEGF-A_{121} that is responsible for the binding of VEGF-A_{165} to NRP-1 (3, 5). Pretreatment of BAEC with GST-Ex7 (up to 10^{-7} M) 15 min prior to stimulation with VEGF-A_{165} (10^{-9} M) reduced PGI_2 synthesis to the level induced by VEGF-A_{121} (Fig. 2*B*). Pretreatment with GST (up to 10^{-7} M), without the exon 7 insert did not alter VEGF-A_{165}-induced PGI_2 synthesis, and neither

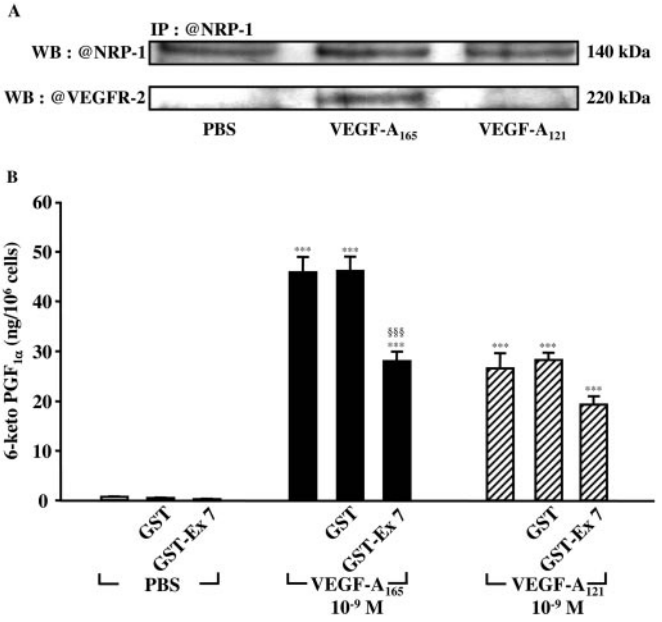

FIG. 2. **NRP-1 coreceptor potentiates PGI_2 synthesis induced by VEGF-A_{165}.** *A*, post-confluent BAEC were stimulated with PBS, VEGF-A_{165}, or VEGF-A_{121} (10^{-9} M) for 7.5 min. Cell lysates were immunoprecipitated with anti-NRP-1 IgG. NRP-1 protein expression in BAEC was detected by immunoblotting. The membranes were stripped, and the detection of VEGFR-2 protein expression was performed by reprobing the membranes with anti-VEGFR-2 IgG. *WB*, Western blotting; *IP*, immunoprecipitation. *B*, post-confluent BAEC were pretreated with the NRP-1 antagonist GST-Ex7 (10^{-7} M) or its carrier GST (10^{-7} M) 15 min prior to stimulation with PBS, VEGF-A_{165}, or VEGF-A_{121} (10^{-9} M) for 15 min. ***, $p < 0.001$ *versus* PBS; §§§, $p < 0.001$ *versus* VEGF-A_{165}.

GST-Ex7 nor GST (up to 10^{-7} M) altered significantly the basal level of PGI_2 synthesis or VEGF-A_{121}-induced PGI_2 synthesis (Fig. 2*B*).

Because VEGF-A_{165} and VEGF-A_{121} 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 PGI_2 synthesis. Treatment with PlGF, VEGF-B, or VEGF-C (10^{-11}-10^{-8} M) for 15 min did not promote the release of PGI_2 (Fig. 3, *A–C*). To assess whether a parallel activation of VEGFR-1 and VEGFR-2 homodimers may induce PGI_2 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 PGI_2 synthesis (Fig. 3*D*).

Phosphorylation of VEGF Receptors by Corresponding VEGF Analogs—Because VEGF-A_{165} and VEGF-A_{121} were the only VEGF analogs capable of mediating endothelial PGI_2 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. VEGFR-1 and VEGFR-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-A_{165} and VEGF-A_{121} (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-

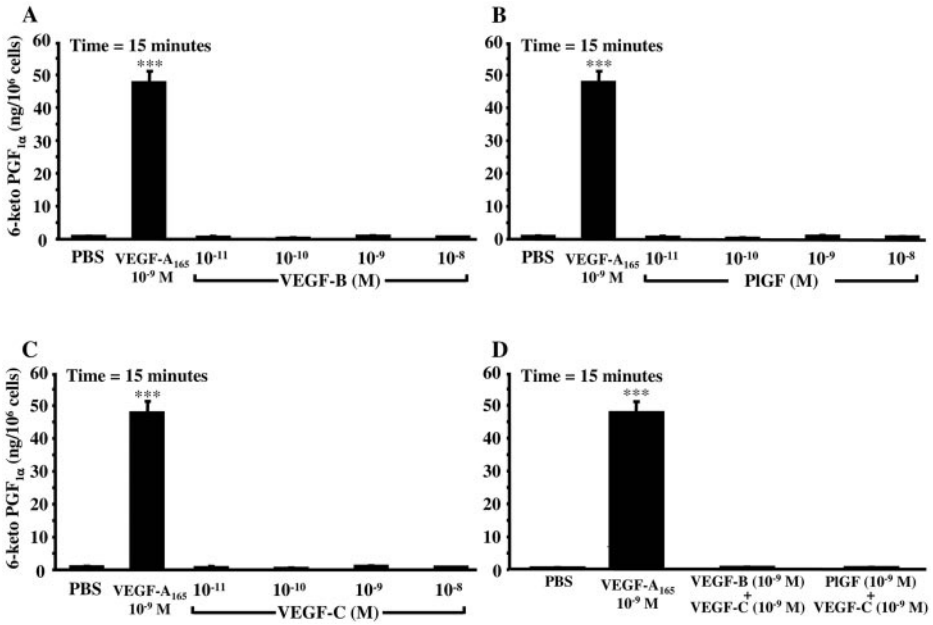

FIG. 3. **Effect of VEGFR-1 and VEGFR-2 agonists on PGI_2 synthesis.** Post-confluent BAEC were stimulated with PBS, VEGFR-1 (VEGF-B and PlGF) (*A* and *B*), or VEGFR-2 (VEGF-C) agonists (10^{-11}-10^{-8} M) alone (*C*) or combined (*D*) for 15 min. BAEC were treated as well with VEGF-A_{165} (10^{-9} M) as positive control. ***, $p < 0.001$ *versus* PBS-treated cells.

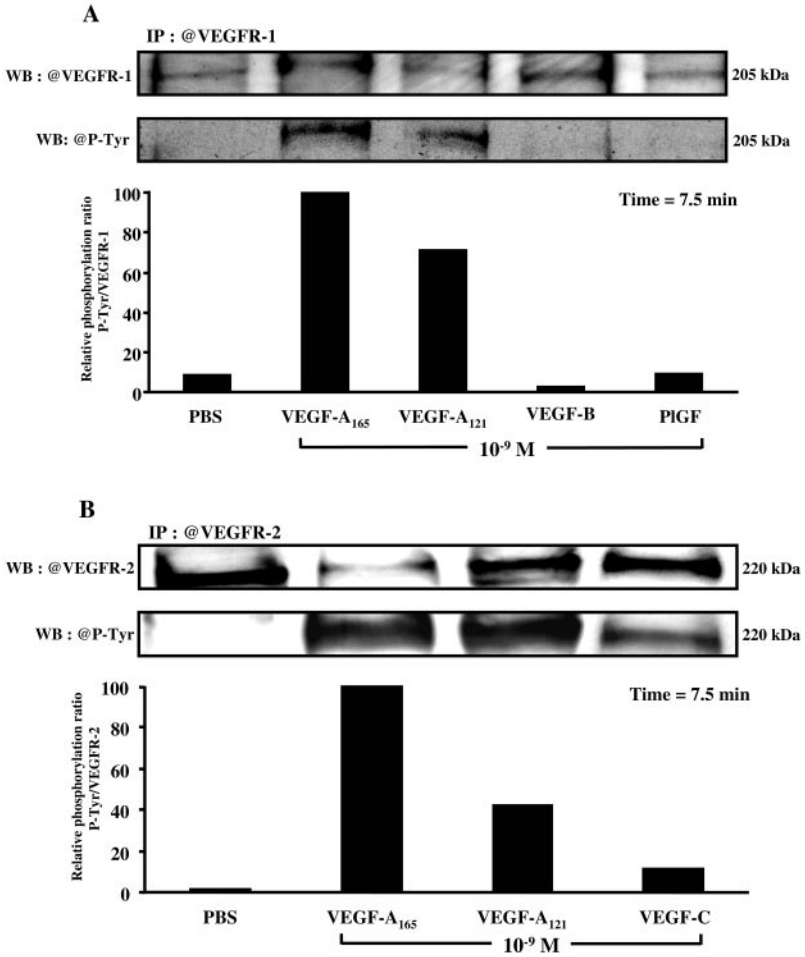

FIG. 4. **Expression and regulation of VEGF receptors by corresponding agonists.** Post-confluent BAEC were stimulated with PBS, VEGF-A_{165}, VEGF-A_{121}, PlGF, VEGF-B, or VEGF-C (10^{-9} M) for 7.5 min. Cell lysates were immunoprecipitated with VEGFR-1 IgG (*A*, 3 mg of total proteins) or VEGFR-2 IgG (*B*, 1 mg of total proteins). VEGFR-1 and VEGFR-2 protein expression in BAEC was detected by immunoblotting (*upper bands*). Membranes were subsequently stripped using Re-Blot Plus Strong stripping solution, and the detection of VEGFR-1 (*A*) and VEGFR-2 (*B*) phosphorylation was performed with an anti-phosphotyrosine IgG (*lower bands*). The phosphorylation level of each receptor was expressed as a function of the protein expression level of VEGFR-1 and VEGFR-2 and normalized with VEGF-A_{165}-treated cells. *WB*, Western blotting; *IP*, immunoprecipitation.

ment with PlGF or VEGF-B did not increase VEGFR-1 phosphorylation (Fig. 4*A*, *lower panel*). Although we could not detect 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 translocation (data not shown and as described previously) (5). Next, we assessed the capacity of VEGF-A_{165}, VEGF-A_{121}, 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. 4*B*, *lower panel*).

PGI_2 Synthesis Requires VEGFR-1 and VEGFR-2 Heterodimerization—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 PGI_2 synthesis. Consequently, we speculated that VEGF-A isoforms

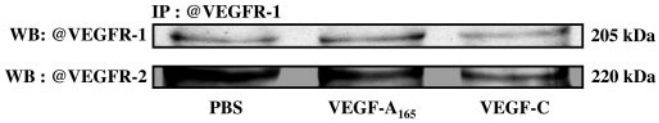

FIG. 5. **Heterodimerization capacity of VEGFR-1 and VEGFR-2 subunits.** Post-confluent BAEC were stimulated with PBS, VEGF-A_{165}, or VEGF-C (10^{-9} M) for 7.5 min. Cell lysates were immunoprecipitated with VEGFR-1 IgG (3 mg of total proteins). VEGFR-1 protein expression in BAEC was detected by immunoblotting. The membranes were stripped, and the detection of VEGFR-2 protein expression was performed by reprobing the membranes with anti-VEGFR-2 IgG. *WB*, Western blotting; *IP*, immunoprecipitation.

induce PGI_2 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-A_{165} or VEGF-C did not modulate VEGFR-1/R-2 dimerization (Fig. 5). Next, to demonstrate that PGI_2 synthesis mediated by VEGF-A_{165} and VEGF-A_{121} 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 (5×10^{-7} 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 PGI_2 mediated by VEGF-A_{165} and, by 73 and 62%, the synthesis of PGI_2 mediated by VEGF-A_{121}, respectively (Fig. 6*A*). As negative control, BAEC were treated with a scrambled oligomer sequence, which did not decrease significantly the level of PGI_2 synthesis mediated by VEGF-A_{165} and VEGF-A_{121}. In addition, the treatment of BAEC with antisense or scrambled oligomers did not affect the basal level of PGI_2 synthesis in PBS-treated cells (Fig. 6*A*).

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} M, IC_{50} = 2.0 and 0.1 × 10^{-6} M, respectively) (18) abrogated by 100 and 90% the synthesis of PGI_2 mediated by VEGF-A_{165} and VEGF-A_{121}, respectively. Similarly, the blockade of VEGFR-2 activity with SU1498 (selective VEGFR-2 inhibitor; 5×10^{-6} M; $IC_{50} = 7 \times 10^{-7}$ M) (5, 19) was sufficient as well to abrogate by 96 and 95% the synthesis of PGI_2 mediated by VEGF-A_{165} and VEGF-A_{121}, respectively (Fig. 6*B*).

To verify the selectivity of VEGF receptor inhibitors, we assessed their corresponding inhibitory effect on VEGFR-1 and VEGFR-2 phosphorylation mediated by VEGF-A_{165}. Pretreatment of BAEC with SU1498 (5×10^{-6} M) 15 min prior to stimulation with VEGF-A_{165} (10^{-9} 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-A_{165} (Fig. 7) by 100 and 83%.

Cell Signaling Pathways by Which VEGF-A_{165} Induces PGI_2 Synthesis—In previous studies, we have shown that VEGF-A_{165} induces PAF synthesis upon the activation of the VEGFR-2/R-2·NRP-1 complex and requires the activation of both p38 and p42/44 MAPKs and subsequent activation of $sPLA_2$, type V ($sPLA_2$-V) (20, 21). Because VEGF-A_{165} induces PGI_2 synthesis upon VEGFR-1/R-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 PGI_2 synthesis. Pretreatment of BAEC with p38 MAPK inhibitor SB203580 (10^{-5} M; $IC_{50} = 6 \times 10^{-7}$ M) (22) did not prevent PGI_2 synthesis mediated by VEGF-A_{165} (10^{-9} M). However, pretreatment with MEK inhibitor PD98059 (10^{-5} M; $IC_{50} = 10^{-6}$ M) (23), which prevents p42/44 MAPK phosphorylation, abrogated the synthesis of PGI_2 mediated by VEGF-A_{165} (Fig. 8). BAEC then were pretreated with PLA_2 inhibitors. Pretreatment with a broad range inhibitor of $sPLA_2$, scalaradial (10^{-5} M) (21), which is capable of preventing VEGF-A_{165}-mediated PAF synthesis, did not reduce the release of PGI_2 significantly, whereas a pretreatment with $cPLA_2$ inhibitor $AACOCF_3$ (5×10^{-5} M, $IC_{50} = 10^{-7}$ M) (24) inhibited the release of PGI_2 mediated by VEGF-A_{165} (Fig. 8) by 86%. Finally, a pretreatment of BAEC, either with a non-selective inhibitor of COX-1 and COX-2 (indomethacin; 10^{-5} M, IC_{50} = 0.1 and 6×10^{-6} M, respectively) (25) or with a selective inhibitor of PGI_2 synthase (tranylcypromine; 3×10^{-3} M) (26), abrogated completely the synthesis of PGI_2 mediated by VEGF-A_{165} (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).

Denitrosylation of Prostacyclin Synthase Is Required to Promote PGI_2—Under quiescent state, PGI_2 synthase is *S*-nitrosylated, thus preventing its capacity to convert its substrate, PGH_2, into PGI_2 (27). In a previous study, we have shown that VEGF-A_{165} and VEGF-C are capable of mediating PAF synthesis upon VEGFR-2 homodimer activation (4, 16). In the current study, we also observed that VEGF-A_{165} and VEGF-C are both capable to promote the phosphorylation p42/44 MAPK and $cPLA_2$ (data not shown), which are essential to promote arachidonic acid release (8, 28). However, VEGF-C as opposed to VEGF-A_{165} cannot induce PGI_2 synthesis. Consequently, we hypothesized that VEGF-C may not be capable of promoting the *S*-denitrosylation of PGI_2 synthase, which is required for PGI_2 production (27, 29).

Thus, we assessed, by Western blot analysis, the capacity of VEGF-A_{165} and VEGF-C to regulate the level of PGI_2 synthase nitrosylation. Treatment with VEGF-A_{165} (10^{-9} M) induced a rapid/immediate *S*-denitrosylation of PGI_2 synthase by 30%, which returned to its basal nitrosylated state after 15 min of treatment. At the opposite, treatment with VEGF-C (10^{-9} M) had no such effect (Fig. 9).

DISCUSSION

Previous studies reported the capacity of VEGF-A_{165} to promote PGI_2 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-A_{165} and VEGF-A_{121}) were able to promote an acute endothelial PGI_2 synthesis, whereas the stimulation of BAEC with selective VEGFR-1 (PlGF or VEGF-B) or VEGFR-2 (VEGF-C) agonists, alone or combined, had no such effect. In addition, VEGF-A_{165} was approximately twice potent as VEGF-A_{121} to promote PGI_2 synthesis. Pretreatment of ECs with a GST-Ex7 fusion protein, which prevents the binding of VEGF-A_{165} to NRP-1, reduced the capacity of VEGF-A_{165} to promote PGI_2 synthesis to the level mediated by VEGF-A_{121}, 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 PGI_2 synthesis and depends on the activation of the VEGFR-1/R-2 heterodimer. Furthermore, NRP-1 potentiates the capacity of VEGF-A_{165} to promote PGI_2 synthesis.

To assess the requirement of VEGFR-1/R-2 activation for promoting PGI_2 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

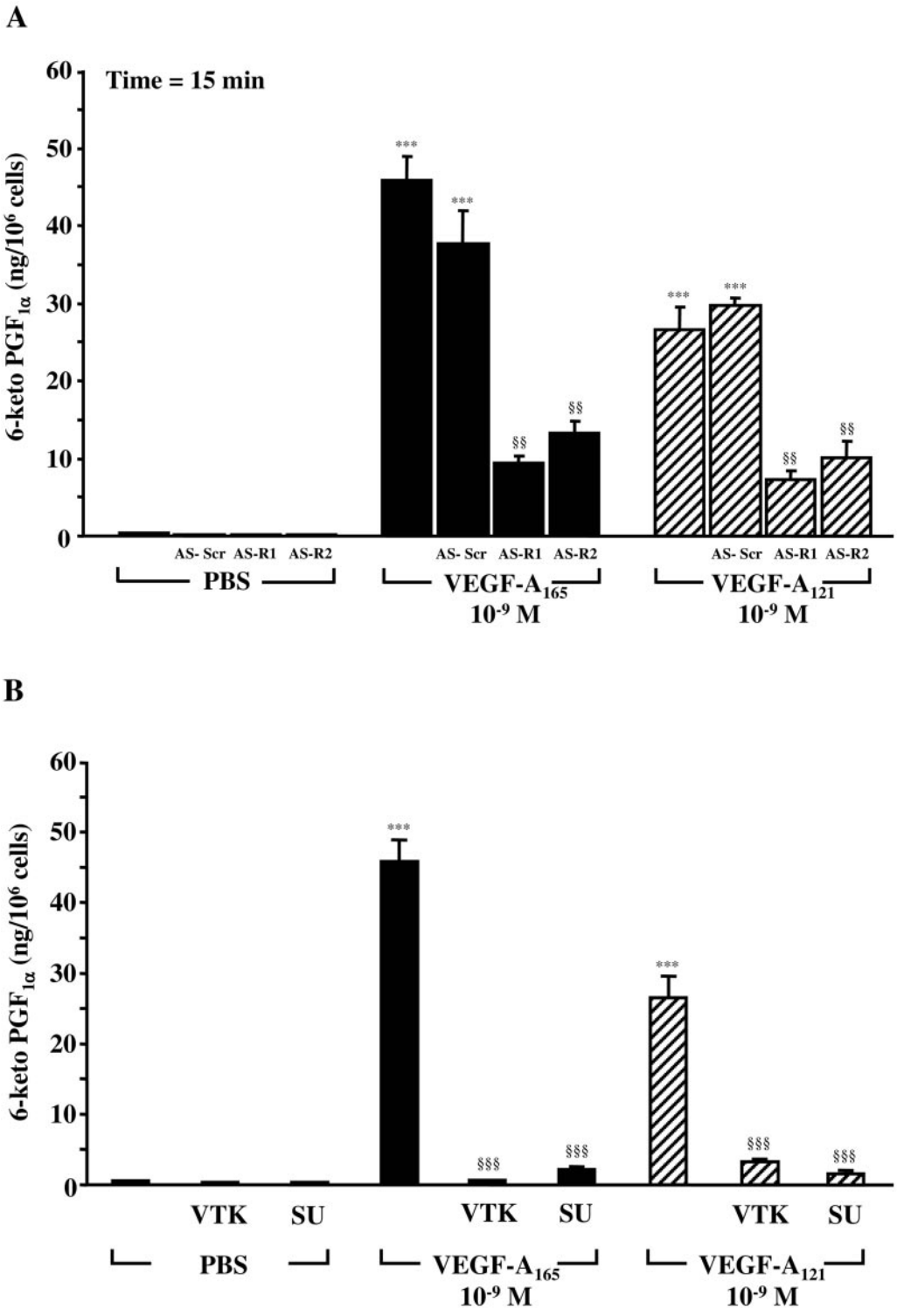

FIG. 6. **Contribution of VEGF receptors on PGI_2 synthesis induced by VEGF-A isoforms.** *A*, BAEC were plated at 5×10^5 cells/well in 6-well plates. Antisense oligomers targeting VEGFR-1 (*AS-R1*) or VEGFR-2 (*AS-R2*) mRNA or a scrambled oligomer (*As-Scr*) were added daily (5×10^{-7} M) up to 3 days 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. PGI_2 synthesis was assessed by quantifying its stable metabolite (6-keto $PGF_{1\alpha}$) by ELISA. ***, $p < 0.001$ *versus* PBS-treated cells; §§, $p < 0.01$, and §§§, $p < 0.001$ *versus* cells treated with VEGF-A isoforms.

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 PGI_2 mediated by VEGF-A_{165} and VEGF-A_{121} (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 VEGFR-2 activation by VEGF-A isoforms and PGI_2 synthesis. Together, these data demonstrate that the activation of both receptors under heterodimeric state (VEGFR-1/R-2) is required for PGI_2 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,

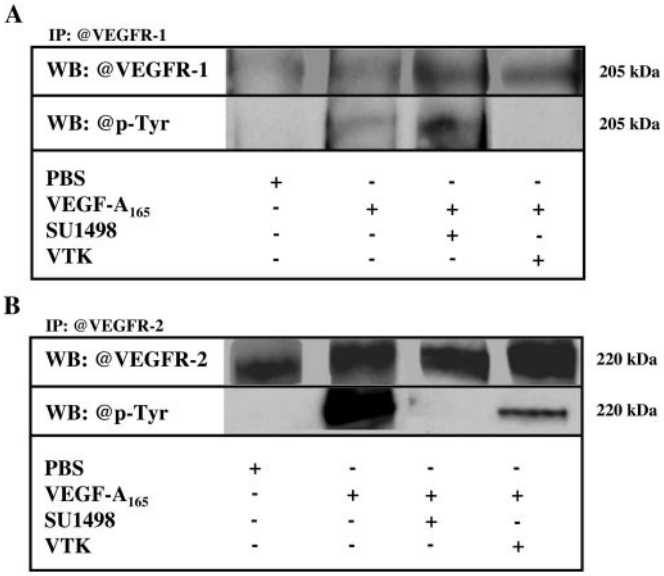

FIG. 7. **Regulation of VEGF receptors phosphorylation by selective inhibitors.** Post-confluent BAEC were pretreated with VTK (10^{-5} M) or SU1498 (5×10^{-6} M) 15 min prior to stimulation with PBS and VEGF-A_{165} (10^{-9} M) for 7.5 min. Cell lysates were immunoprecipitated with VEGFR-1 (*A*) or VEGFR-2 (*B*) IgG. Western blot analyses for VEGFR-1 (*A*) or VEGFR-2 (*B*) protein expression were performed (*upper bands*), and then the membranes were stripped and the detection of VEGFR-1 (*A*) and VEGFR-2 (*B*) phosphorylation was performed with an anti-phosphotyrosine IgG (*lower bands*). *WB*, Western blotting; *IP*, immunoprecipitation.

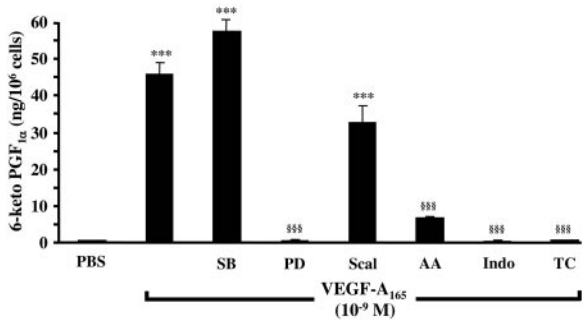

FIG. 8. **Cell signaling pathway by which VEGF-A_{165} promotes PGI_2 synthesis.** Post-confluent BAEC were pretreated with selective inhibitors of p38 MAPK (SB203580 (*SB*), 10^{-5} M), MEK (PD98059 (*PD*), 10^{-5} M), $sPLA_2$-V (scalaradial (*Scal*), 10^{-5} M), or $cPLA_2$ ($AACOCF_3$ (*AA*), 5×10^{-5} M), COX-1/-2 (indomethacin (*Indo*), 10^{-5} M), or PGI_2 synthase (tranylcypromine (*TC*), 3×10^{-3} M) 15 min prior to stimulation with VEGF-A_{165} (10^{-9} M) for 15 min. Synthesized PGI_2 was assessed by quantifying its stable metabolite (6-keto $PGF_{1\alpha}$) by ELISA. ***, $p < 0.001$ *versus* PBS-treated cells; §§§, $p < 0.001$ *versus* VEGF-A_{165}-treated cells.

because the blockade of VEGFR-1 or VEGFR-2 expression and corresponding activation were sufficient to prevent PGI_2 synthesis. Furthermore, parallel activation of VEGFR-1/R-1 and VEGFR-2/R-2 by selective agonists did not promote PGI_2 synthesis.

Although we detected the phosphorylation of VEGFR-1 mediated by VEGF-A isoforms, we could not detect its phosphorylation upon a treatment with PlGF and VEGF-B. Consequently, one might argue that this could explain why PlGF and VEGF-B were unable to promote PGI_2 synthesis. However, this hypothesis does not stand because we observed in a different study that PlGF 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 (5). Our data are also in agreement with previous reports that have shown that PlGF 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 PGI_2 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 PGI_2 synthesis.

Our observations are also supported by recent studies that have demonstrated that, in native unstimulated and VEGF-A_{165}-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 provided 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-A_{165} resulted in signal transduction with different efficiencies and biological activities (40). For instance, migration of PAEC co-expressing VEGFR-1/R-2 toward VEGF-A_{165} 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-A_{165} can induce a strong phosphorylation of VEGFR-1 tyrosine residue Tyr-1213 and to lesser extent Tyr-1242 and Tyr-1333, whereas PlGF 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-A_{165} and its analogs. Because VEGF-A_{165} and VEGF-A_{121} are the only VEGF analogs capable to bind VEGFR-1/R-2 heterodimer, they might provide an exclusive transphosphorylation of VEGFR-1 and VEGR-2 subunits that appears to be essential to govern cell signaling leading to PGI_2 synthesis.

In previous studies, we have shown that VEGF-A_{165} 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 $sPLA_2$-V (20, 21). Interestingly, the stimulation of VEGFR-1 did not promote PAF synthesis and was not required to support VEGF-A_{165}-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 PGI_2 synthesis mediated by VEGF-A_{165}, we investigated the cell signaling pathways involved in PGI_2 synthesis. Previous studies highlighted the contribution of p42/44 and/or p38 MAPK on PGI_2 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

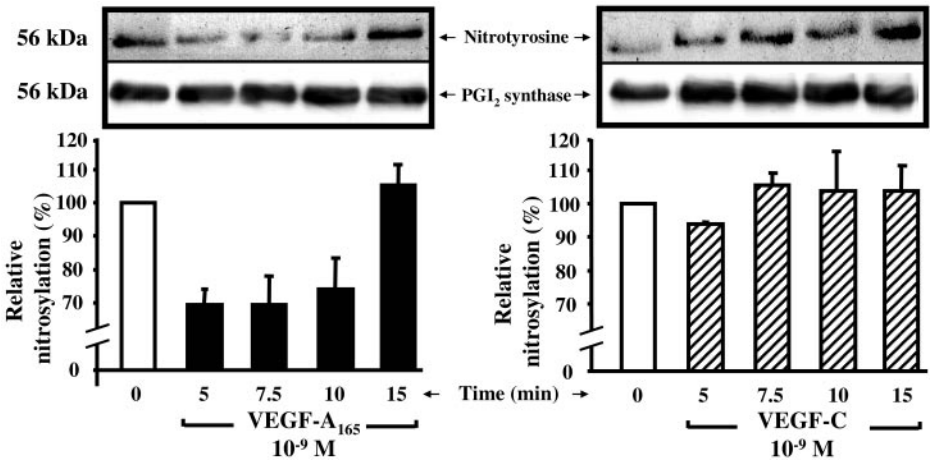

FIG. 9. ***S*-Nitrosylation of PGI_2 synthase in the presence of VEGF-A_{165} and VEGF-C.** Post-confluent BAEC were stimulated time-dependently with PBS, VEGF-A_{165}, or VEGF-C (10^{-9} M). Cell lysates (500 μg of total proteins) were immunoprecipitated with PGI_2 synthase IgG. Western blot analyses were performed with an anti-nitrotyrosine IgG to assess the *S*-nitrosylation level of PGI_2 synthase, and then the membranes were stripped and reprobed with an anti-PGI_2 synthase IgG as described above. The nitrosylation level of PGI_2 synthase upon stimulation with VEGF-A_{165} or VEGF-C was expressed as a function of the protein expression level of PGI_2 synthase, and the results were normalized to PBS-treated cells.

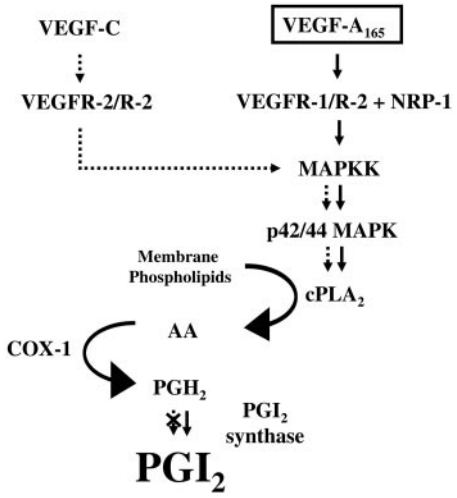

FIG. 10. **Proposed signaling pathway by which VEGF-A_{165} mediates PGI_2 synthesis.** PGI_2 synthesis induced by VEGF-A_{165} requires the heterodimerization of VEGFR-1/R-2 subunits and is potentiated by the contribution of NRP-1 coreceptor. Such a complex that can activate downstream effectors (namely p42/44 MAPK and $cPLA_2$) provides the release of arachidonic acid, which can be converted upon the activation of COX-1 and PGI_2 synthase into PGI_2. On the other hand, the activation of VEGFR-2/R-2 homodimer by VEGF-C can promote similar cell signaling up to the release of arachidonic acid; however, VEGF-C is unable to promote *S*-denitrosylation of PGI_2 synthase, which is essential for PGI_2 synthesis.

MAPK activation prevented the induction PGI_2 synthesis mediated by VEGF-A_{165}, whereas the blockade of p38 MAPK activity had no such effect. We previously reported that PAF synthesis mediated by VEGF-A_{165} requires the activation of $sPLA_2$ as opposed to $cPLA_2$. However, because it is well established that p42/44 MAPK can promote the phosphorylation of $cPLA_2$ (8, 46) and that p42/44 MAPK activation is essential for PAF synthesis (20), we assessed the contribution of both PLA_2 on PGI_2 synthesis. The blockade of $cPLA_2$ prevented completely the synthesis of PGI_2, whereas the inhibition of $sPLA_2$ induced a non-significant decrease of PGI_2 synthesis.

Because VEGF-C is also capable of promoting VEGFR-2 activation and PAF synthesis but not PGI_2 synthesis, we investigated by Western blot analyses its capacity to induce the phosphorylation p42/44 MAPK and $cPLA_2$. In both cases, VEGF-C promoted the activation of these enzymes, suggesting that the activation of these enzymes is not the rate-limiting step for the incapacity of VEGF-C to mediate PGI_2 synthesis. The activation of $cPLA_2$ catalyzes the cleavage of arachidonic acid from the *sn*-2 position of phospholipids, which is then converted into PGH_2 by COX-1 and/or COX-2, which serves as substrate for PGI_2 synthase and PGI_2 production (11–13, 41). By Western blot analysis, we confirmed the presence of constitutive COX-1, whereas COX-2 was not present. This is in agreement with previous reports that have shown in quiescent and unstimulated ECs that COX-2 isoform is not present and that its up-regulation by diverse cytokines including VEGF is achieved at least two hours after stimulation (12, 14, 47–49). Therefore, only COX-1 isoform was involved and its contribution was essential since its blockade with indomethacin abrogated PGI_2 synthesis. Furthermore, because COX-1 activity is not repressed by VEGF isoforms (14) and because there is no alternative pathway for PGI_2 synthesis, this brings us to suggest that COX-1 activation cannot be responsible for the difference between VEGF-C and VEGF-A_{165} to promote PGI_2 synthesis. Thus, the rate-limiting step can be due to a possible difference in their capacity to promote PGI_2 synthase activity. Recently, a new post-translational modification was found for the different isoforms of nitric-oxide synthase and that the nitrosylation of nitric-oxide synthase provides a negative regulatory mechanism on nitric oxide production (50, 51). The same nitrosylation mechanism is also observed for the regulation of PGI_2 synthase activity upon the *S*-nitrosylation of the tyrosine 430 residue (52). In our study, we observed in quiescent non-stimulated EC that the PGI_2 synthase was constitutively nitrosylated, which is concordant with the very low concentration of prostacyclin in BAEC. Treatment with VEGF-A_{165} induced a parallel rapid and transient denitrosylation of PGI_2 synthase and PGI_2 synthesis, whereas a treatment with VEGF-C induced neither PGI_2 synthase denitrosylation nor PGI_2 synthesis. The specific mechanism by which PGI_2 synthase denitrosylation occurs remains to be identified. However, recent studies proposed the existence of a nitrase-denitrase tyrosine activity, which is regulating the nitrosylation-denitrosylation equilibrium of selective proteins such as PGI_2 synthase (53, 54). Consequently, our data suggest that the only difference in the signaling pathways leading to PGI_2 synthesis of VEGF-A_{165} as opposed to VEGF-C is the capacity of VEGF-A_{165} to promote, upon the activation of VEGFR-1/R-2 heterodimer, a rapid and transient of PGI_2 synthase denitrosylation and PGI_2 release (Fig. 10).

In conclusion, we observed for the first time the necessity for VEGFR-1/R-2 heterodimer activation to promote a selective biological activity in occurring PGI_2 synthesis. We have also observed that, even if VEGF-C and VEGF-A_{165} are capable of activating the same signaling pathways leading to endothelial arachidonic acid release, they showed a different capacity to promote PGI_2 synthase denitrosylation and therefore PGI_2 synthesis. Such a difference would be attributable to the exclusive capacity of VEGF-A isoforms to activate VEGFR-1/R-2 het-

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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