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Angiopoietin-mediated endothelial P-selectin translocation: cell signaling mechanisms

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Abstract: Recently identified, angiopoietin-1 (Ang1) and -2 (Ang2) bind to the tyrosine kinase receptor Tie2 and contribute to orchestrate blood vessel formation during angiogenesis. Ang1 mediates vessel maturation and integrity by favoring the recruitment of pericytes and smooth muscle cells. Ang2, initially identified as a Tie2 antagonist, may under certain circumstances, induce Tie2 phosphorylation and biological activities. As inflammation exists in a mutually dependent association with angiogenesis, we sought to determine if Ang1 and/or Ang2 could modulate proinflammatory activities, namely P-selectin translocation, in bovine aortic endothelial cells (EC) and dissect the mechanisms implicated. P-selectin, an adhesion molecule found in the Weibel-Palade bodies of EC, is translocated rapidly to the cell surface upon EC activation during inflammatory processes. Herein, we report that Ang1 and Ang2 (1 nM) are capable of mediating a rapid Tie2 phosphorylation as well as a rapid and transient endothelial P-selectin translocation maximal within 7.5 min (125% and 100% increase, respectively, over control values). In addition, we demonstrate for the first time that angiopoietin-mediated endothelial P-selectin translocation is calcium-dependent and regulated through phospholipase C-γ activation. *J. Leukoc. Biol.* 83: 000–000; 2008.

Key Words: *Tie2 receptor · adhesion molecules · angiogenic factors*

INTRODUCTION

Angiogenesis, the formation of new blood vessels from a preexisting vasculature, is a tightly regulated process, requiring the action of various growth factors, namely, vascular endothelial growth factor (VEGF) and angiopoietins (ref. [1] for review). Angiogenesis plays a critical role in several pathological conditions, including atherosclerosis, proliferative retinopathies, and tumor growth [1]. It has been reported that inflammation precedes and accompanies pathological angiogenesis, as evidenced by increased vascular permeability as well as monocyte/macrophage and neutrophil recruitment at angiogenic sites [2]. During inflammatory processes, newly formed vessels supply inflamed tissue with oxygen and nutrients and facilitate the transport of inflammatory cells.

Recently identified, the angiopoietins (Ang1 and Ang2) are defined as ligands for the tyrosine kinase receptor Tie2 [3, 4], to which they bind with similar specificity and affinity [3, 4]. Ang1 has been characterized as a Tie2 agonist, having the capacity to stabilize and promote the maturation of unstable vessels in the presence of VEGF-A 165 isoform (VEGF-A_{165}) [5]. Conversely, Ang2 was described initially as a natural endogenous Tie2 antagonist, thereby destabilizing existing vessels prior to VEGF-A_{165}-induced angiogenic sprouting [4]. However, under certain circumstances, Ang2 may induce Tie2 phosphorylation, intracellular cell signaling events, and biological activities such as endothelial cell (EC) migration and platelet-activating factor (PAF) synthesis, neutrophil activation, vascular permeability, and in vitro tubule capillary-like formation [6–15].

Neutrophils are the first cells recruited to inflammatory sites, providing cytokines, growth factors, and proteolytic enzymes, which contribute to trigger and support angiogenic activities [16, 17]. The recruitment of neutrophils implies overlapping succession of adhesive events encompassing neutrophil tethering, rolling, and firm adhesion onto EC. These processes require the interaction of adhesion molecules located on the surface of EC and neutrophils. Stimulation of EC with inflammatory mediators can promote a rapid and transient translocation of P-selectin contained in Weibel-Palade bodies (WPB) to the cell surface [18–20]. P-selectin is then able to interact with its high-affinity counter-receptor, P-selectin-glycoprotein-ligand-1, expressed on neutrophils and promote their rolling and transient adhesion [21]. Inflammatory mediators may also lead to an equivalent rapid and transient synthesis of PAF by EC and/or neutrophils. Newly synthesized PAF can then bind to its receptor expressed on neutrophils and induce a rapid, functional up-regulation of the β_2-integrin complex (CD11/CD18), favoring the binding to its endothelial counter-receptor, ICAM-1 and ICAM-2. This latter interaction increases the adhesion of neutrophils onto activated EC, which is critical in the initiation of the inflammatory process at injury sites (refs. [19, 22, 23] for review).

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We have reported that VEGF-A_{165} inflammatory effects are mediated through the synthesis of PAF by EC [24], which contribute to the induction of endothelial P-selectin translocation and neutrophil adhesion onto activated EC [20]. Furthermore, our laboratory demonstrated that Ang1 and Ang2 are capable of promoting endothelial P-selectin translocation and neutrophil adhesion onto EC [12]. As angiopoietins act in concert with VEGF to modulate vascular plasticity during postnatal neovascularization [25], we therefore sought to investigate the cellular mechanisms implicated in angiopoietin-mediated endothelial P-selectin translocation and assess the role of PAF in this process.

MATERIALS AND METHODS

Reagents

BN 52021, CV-3988, PD98059, SB203580, Wortmannin, U73122, Calphostin C, and W-7 were purchased from Calbiochem (La Jolla, CA, USA). BAPTA-AM was purchased from Sigma (Oakville, ON, Canada). LAU 8080 was a generous gift from Dr. Nicolas G. Bazan (Department of Ophtalmology and Neuroscience Center of Excellence, Louisiana State University, New Orleans, LA, USA).

Cell culture

EC were isolated from fresh bovine aortic EC (BAEC), seeded in flat-bottom 96-well plates (Fisher Scientific, Nepean, ON, Canada), and cultured in DMEM (Life Technologies, Burlington, ON, Canada) containing 5% FBS (Medicorp Inc., Montreal, QC, Canada) and 1% antibiotics (penicillin and streptomycin, Sigma). BAEC were characterized as described previously [24, 26] and used between Passages 2 and 5.

Western blot analysis

Tie2 phosphorylation

Confluent BAEC in 100 mm culture plates were serum-starved in DMEM with antibiotics overnight, rinsed with HBSS (Life Technologies), and then stimulated in a solution of HBSS/HEPES (10 mM, pH 7.4), BSA (1 mg/mL, Sigma), and $CaCl_2$ (1 mM) containing Ang1 or Ang2 (1 nM, R&D Systems, Minneapolis, MN, USA) at 37°C. Cells were solubilized with lysis buffer and scraped, and the protein concentration was determined by the Bradford assay. Cell lysates were immunoprecipitated with rabbit polyclonal anti-mouse Tie2 IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and separated by SDS-PAGE. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and probed with mouse monoclonal antiphosphotyrosine IgG (clone 4G10; 1:1000 dilution, Upstate Biotechnology Inc., Lake Placid, NY, USA). Membranes were stripped using Re-Blot Plus Strong stripping solution (Chemicon International, Temecula, CA, USA) and reprobed with rabbit polyclonal anti-mouse Tie2 IgG (1:1000 dilution, Santa Cruz Biotechnology). Bands were visualized using LumiGlo™ (New England Biolabs, Pickering, ON, Canada). Band OD was determined using Quantity One software (Bio-Rad, Mississauga, ON, Canada).

Phospholipase C-γ (PLC-γ) activation

In another series of experiments, BAEC were pretreated for 15 min with a selective inhibitor of PLC-γ (U73122; 10 μM, Calbiochem) prior to stimulation with VEGF-A_{165} (1 nM, PeproTech Inc., Rocky Hill, NJ, USA), Ang1 or Ang2 (1 nM) for 7.5 min. Cell lysates were separated by SDS-PAGE, and proteins were transferred onto a PVDF membrane. To determine PLC-γ activation, cell lysates were immunoprecipitated with rabbit polyclonal anti-bovine PLC-γ IgG (Santa Cruz Biotechnology) and separated by SDS-PAGE. Proteins were transferred onto a PVDF membrane and probed with mouse monoclonal antiphosphotyrosine IgG (clone 4G10; 1:1000 dilution, Upstate Biotechnology Inc.). Membranes were subsequently stripped and reprobed with rabbit polyclonal anti-human PLC-γ IgG (New England Biolabs) to visualize the total protein expression.

PLC-γ, p38 MAPK, p42/44 MAPK, PI-3K/Akt activation

In another series of experiments, BAEC were pretreated for 15 min with selective inhibitors of p38 MAPK (SB203580, 10 μM), MEK (MAPKK; PD98059, 10 μM), or PI-3K/Akt (Wortmannin, 500 nM, Calbiochem) prior to stimulation with Ang1 or Ang2 (1 nM). As positive control, BAEC were treated with VEGF-A_{165} (1 nM, PeproTech Inc.) for 7.5 min after pretreatment with the aforementioned pathway inhibitors. Cell lysates were separated by SDS-PAGE, and proteins were transferred onto a PVDF membrane. Activation of p38, p42/44, and Akt was determined by probing membranes with antibodies for their respective phosphorylated forms (1:1 000 dilution, New England Biolabs). Membranes were stripped and reprobed subsequently with the appropriate antibodies to visualize corresponding total protein expression.

Ang2 ELISA

The content of Ang2 protein in BAEC supernatant and whole cell extract was quantified using a commercial ELISA kit (Quantikine, R&D Systems). Confluent cells grown in six-well plates were serum-starved overnight in DMEM containing antibiotics prior to stimulation with VEGF-A_{165} (1 nM) or Ang1 (0.01–10 nM) in HBSS-HEPES containing $CaCl_2$ (10 mM) for 15 min. Upon stimulation, cell supernatant was collected, and the cells were scraped and gently sonicated in PBS (pH 7.4) in ice. The ELISA protocol was carried out according to the manufacturer's instructions.

Cell surface ELISA

Endothelial P-selectin translocation was measured by cell surface ELISA, as described previously [12, 20], following stimulation of BAEC with Ang1 or -2. Briefly, BAEC were seeded and grown up to 1 day postconfluence in flat-bottom 96-well plates. Prior to stimulation, BAEC were incubated overnight in serum-free DMEM containing 1% antibiotics. BAEC were rinsed with Dulbecco's phosphate-buffered saline (DPBS; Life Technologies) at 37°C and in function of the experiments, pretreated with a DPBS-$CaCl_2$ (10 mM) solution, with or without selective antagonists or inhibitors, 15 min prior to stimulation with angiopoietins or VEGF-A_{165} (1 nM). Reactions were stopped by removing stimulation medium and adding 1% paraformaldehyde for 20 min. Following a rinse with DPBS, cells were incubated with blocking solution (5% BSA in DPBS) for 15 min. Cells were then incubated with rabbit polyclonal anti-human P-selectin IgG (Research Diagnostics Inc., Concord, MA, USA; 1:1000 dilution) for 90 min, rinsed with DPBS, and then incubated with HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnologies; 1:5000 dilution) for 45 min. Peroxidase activity was quantified at 450 nm using a plate reader. Nonspecific binding was assessed by substituting primary antibodies with normal rabbit IgG (Santa Cruz Biotechnologies). As a result of slight variations of basal P-selectin translocation between experiments, data were reported as relative P-selectin translocation [12, 20].

Confocal microscopy

Immunoflorescence

BAEC were grown on glass coverslips, rinsed with DPBS (37°C), stimulated with VEGF-A_{165}, Ang1, Ang2 (1 nM), or PMA (1 μM), and fixed with 1% paraformaldehyde-DPBS solution. Following fixation, the cells were incubated for 60 min in the dark with wheat germ agglutinin (WGA) conjugated to Alexa 488 (1:200 dilution, Invitrogen, Carlsbad, CA, USA) to label the cell surface membrane. Following a rinse with DPBS, cells were incubated with blocking solution (4% normal donkey serum in DPBS) for 15 min. Cells were incubated with rabbit polyclonal anti-human P-selectin antibodies (1:100 dilution, Research Diagnostics Inc.), and nonspecific binding was assessed by substituting primary antibodies with normal rabbit IgG (Santa Cruz Biotechnologies) for 90 min. Cells were rinsed with DPBS and incubated with donkey anti-rabbit conjugated to Alexa 555 IgG (1:600 dilution, Invitrogen) for 60 min. Glass coverslips were mounted using 1,4-diazabicyclo-2-2-2-octane/glycerol (1:5) solution.

Image acquisition, deconvolution, and image rendering

Z stacks of BAEC were acquired with a LSM 510 confocal microscope (Zeiss, Oberkochen, Germany) and saved as LSM files. Donkey anti-rabbit conjugated to Alexa 555 IgG and WGA conjugated to Alexa 488 were visualized using a 543-nm Helium-Neon laser and a 488-nm Argon laser, respectively. A 63×/1.4 Plan-Apochromat objective (Zeiss) was used for magnification. Voxel size is 71 nm × 71 nm × 160 nm (X, Y, and Z). Z stacks were deconvolved with the Huygens Pro 2.6.5a [Scientific Volume Imaging (SVI), Alexanderlaan, The Netherlands] using the maximum likelihood estimation (MLE) algorithm. Signal-to-noise ratios were quantified for each Z stack and added to the MLE algorithm. Point-spread functions (PSFs) were derived from Z stacks of 15 fluorescent (540–560 nm and 500–515 nm) beads of 170 nm in diameter (Invitrogen). PSFs were acquired the same way as the images of interest. Deconvolutions were applied until reaching 0.01% quality change threshold between iterations. Deconvolved Z stacks were saved in the Tiff files format series or ICS files and transferred to the LSM 510 or simulated fluorescence process (SFP) software (SVI), respectively. Front-view transparent projections were made using four slices (0.64 μm total thickness) from their respective Z stacks. A two (2-μm)-wide rectangle volume was extracted from the Z stacks to produce transverse transparent projections, which were executed with the projection tool from the LSM 510 software. The totality of slices from the Z-stacks was used to produce the SFP projections. Final images were saved as Tiff files.

Statistical snalysis

Data are mean ± SEM. Comparisons were made by ANOVA followed by a Bonferroni *t*-test using GraphPad Instat, Version 3.05 (GraphPad Software Inc., San Diego, CA, USA). Data were considered significantly different if values of $P < 0.05$ were observed.

RESULTS

Ang1 and Ang2 activate Tie2

In an initial series of experiments, we assessed the capacity of both angiopoietins (Ang1 and Ang2) to modulate Tie2 phosphorylation in function of time. Treatment of confluent BAEC with Ang1 (1 nM) induced a rapid Tie2 phosphorylation within 7.5 min, corresponding to a 2.2-fold increase upon 15 min of treatment versus control DPBS-treated cells (**Fig. 1A**). Similarly, treatment with Ang2 (1 nM) also induced within 7.5 min Tie2 phosphorylation and corresponded with a 5.1-fold increase following 15 min treatment compared with control, DPBS-treated cells (Fig. 1B).

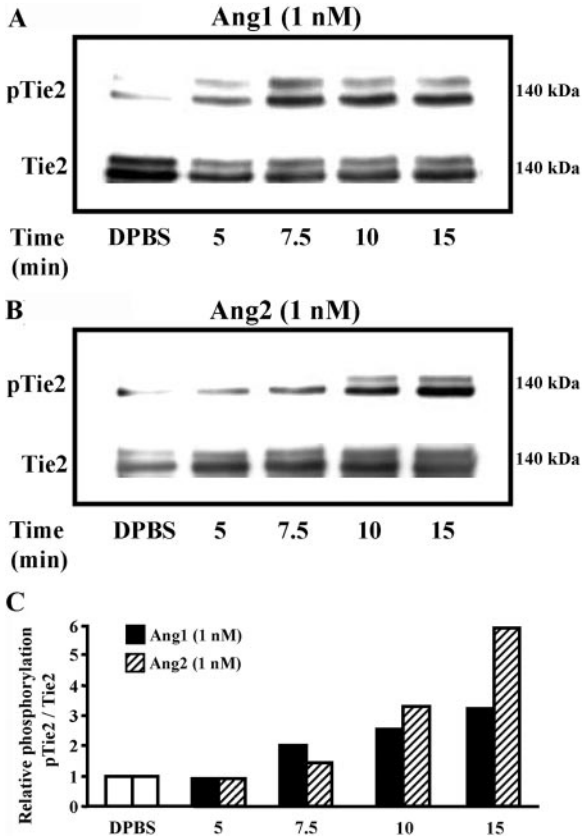

Fig. 1. Ang1 and Ang2 activate Tie2 in a time-dependent manner. Confluent BAEC were treated with Ang1 (A) or Ang2 (B) from 5 min to 15 min. Cell lysates (500 μg) were prepared and immunoprecipitated with rabbit polyclonal anti-mouse Tie2 IgG. Following resolution by SDS-PAGE and transfer, PVDF membranes were probed with mouse monoclonal antiphosphotyrosine IgG, and immunoreactive bands were visualized by chemiluminesence. Membranes were subsequently stripped using ReBlot Plus Strong stripping solution, and Tie2 protein expression was determined by Western blot analysis following incubation with rabbit polyclonal anti-mouse Tie2 IgG. Relative Tie2 phosphorylation (pTie2) over Tie2 total protein expression under various experimental conditions was calculated by normalizing DPBS control OD values to 1 (C).

Ang1 and Ang2 induce P-selectin translocation in BAEC

We have reported previously that VEGF-A_{165} requires endothelial PAF synthesis to promote P-selectin translocation in HUVEC [20]. In addition, we observed that Ang1 and Ang2 are capable of inducing P-selectin translocation in HUVEC and this, in the absence of endothelial PAF synthesis [12]. More recently, we demonstrated that Ang1 and Ang2 are capable of mediating PAF synthesis in BAEC [9], and thus, we sought to determine whether angiopoietins can mediate P-selectin translocation in BAEC and the potential contribution of PAF. Stimulation of BAEC with Ang1 induced endothelial P-selectin translocation in a time (0–15 min)- and concentration-dependent manner (1 pM–5 nM). Peak values (125% increase) were observed within 7.5 min and at 1 nM, as compared with control, DPBS-treated cells (**Fig. 2, A** and **B**). Treatment of BAEC with Ang2 induced endothelial P-selectin translocation in a similar manner (100% increase) within 7.5 min at 1 nM (Fig. 2, C and D). As positive control, BAEC were stimulated with VEGF-A_{165} (1 nM) for 7.5 min, increasing by 200% basal endothelial P-selectin translocation (Fig. 2).

Confocal microscopy was used to confirm the ability of angiopoietins to promote endothelial P-selectin translocation, which to the nonpermeabilized EC surface membrane, was visualized by dual labeling of cell membrane with WGA conjugated to Alexa 488 and with antibodies conjugated to Alexa 555 against P-selectin. A negative control experiment was performed by replacing P-selectin primary antibodies with purified, preimmune rabbit IgG, which did not reveal detectable staining of P-selectin (**Fig. 3, A1–A3**). In control, DPBS-treated BAEC, we observed a basal level of P-selectin at the cell surface membrane (Fig. 3, B1–B3). Treatment with positive controls (PMA, 1 μM; VEGF-A_{165}, 1 nM) or with Ang1 or Ang2 (1 nM) for 7.5 min induced P-selectin translocation along the cytoplasmic membrane (Fig. 3, C1–C3 to F1–F3, respectively).

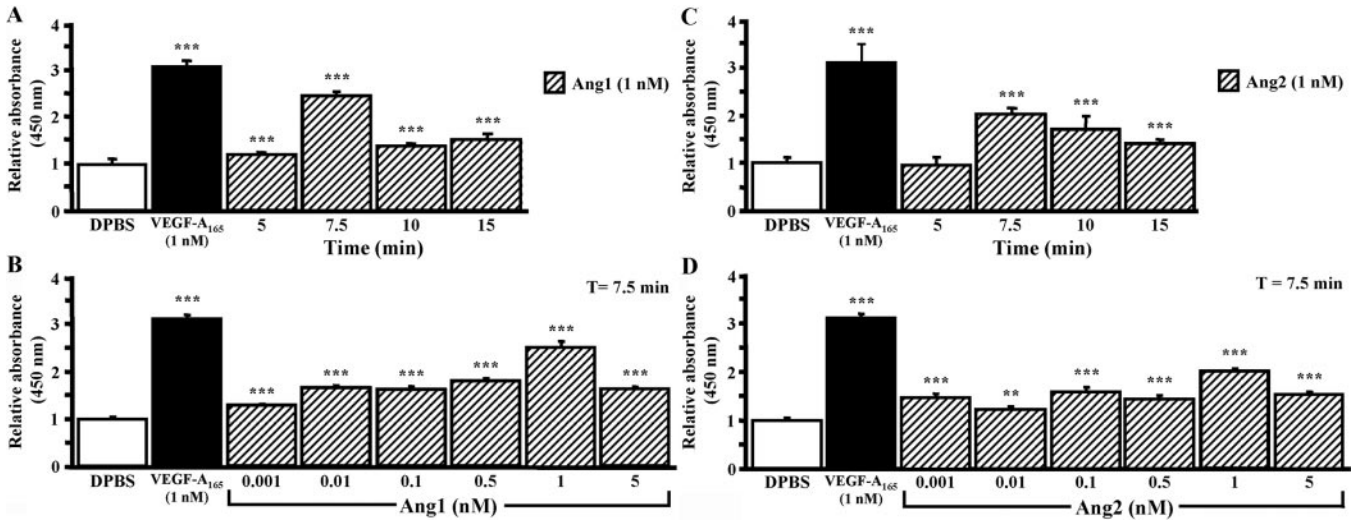

Fig. 2. Ang1 and Ang2 induce endothelial P-selectin translocation in a time- and concentration-dependent manner. Confluent BAEC were treated with Ang1 or Ang2, and P-selectin translocation was measured by cell surface ELISA. Solution buffer (DPBS) was used as control, and the basal levels of P-selectin translocation were normalized to 1. Maximal P-selectin translocation (T) was observed at 7.5 min (A) at a concentration of 1 nM (B). Similarly, Ang2-mediated endothelial P-selectin translocation was maximal at 7.5 min (C) and at 1 nM (D). As positive control, BAEC were stimulated with VEGF-A_{165} (1 nM; 7.5 min). Data are expressed as relative absorbance measured at 450 nm. Values are means ± SEM of P-selectin translocation obtained from at least three independent experiments. ** and ***, $P < 0.01$ and $P < 0.001$, versus DPBS, respectively.

Nonpermeabilized cells were used so as to eliminate the signal emanating from P-selectin located in cytoplasmic WPB and thus, enabling the visualization of P-selectin at the cell surface in front view (Fig. 3, A1–F1) and transverse (Fig. 3, A2–F2) transparent projections. In these projections, P-selectin translocation within the cell surface membrane is detected upon stimulation with angiopoietins and positive controls, as evidenced by the presence of a yellow signal. To visualize the

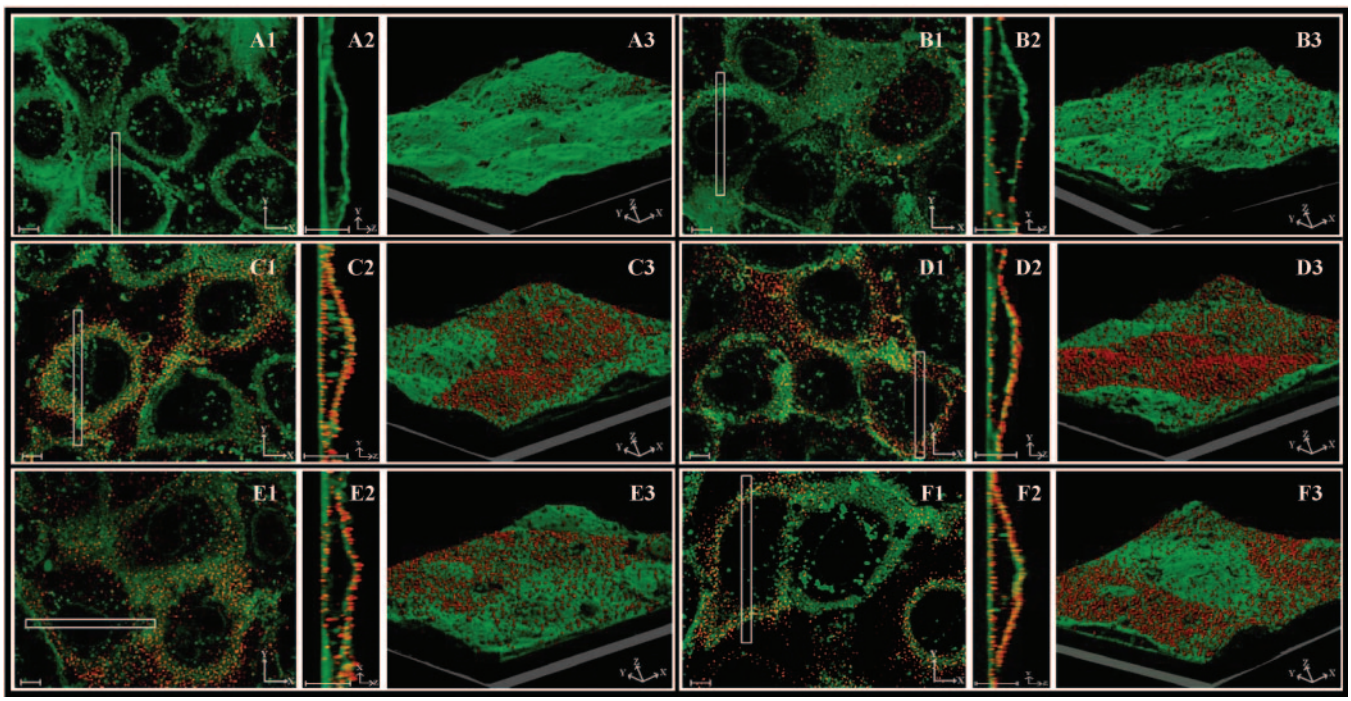

Fig. 3. Ang1 and Ang2 mediate endothelial P-selectin translocation. Confluent BAEC were stimulated with DPBS, PMA (1 μM), VEGF-A_{165}, Ang1, or Ang2 (1 nM) for 7.5 min. Cells were fixed, labeled with antisera, and visualized by confocal microscopy. Cell surface membranes were labeled with WGA conjugated to Alexa 488 and appear in green. P-selectin distribution was detected by using rabbit polyclonal anti-human P-selectin IgG followed by incubation with a secondary antibody conjugated to Alexa 555 IgG and appears in red. The presence of P-selectin within the cell surface membrane appears in yellow. Front view transparent projections (A1–F1) were made using four slices (0.64 μm thick) from respective Z stacks. A 2 μm-wide rectangular volume from the Z stacks was extracted to produce transverse transparent projections (A2–F2), executed using the LSM 510 software. Three-dimensional (3D) representation of the cells (A3–F3) was performed by using the SFP software; projections were created using all the slices of the Z stacks. Original magnification, 63×; digital zoom, 2×; bars, 5 μm.

extracellular P-selectin-translocated domain, confocal images were cumulated to provide a 3D projection of the cells, in which the extracellular P-selectin domain appears in red (Fig. 3, A3–F3).

Regulation of Ang2 release mediated by Ang1 and VEGF-A_{165}

It has been reported that P-selectin and Ang2 are stored separately in WPB from various EC types [27]. In our recent studies, we observed that VEGF, Ang1, and Ang2 are capable of promoting endothelial P-selectin translocation in HUVEC [12, 20], whereas VEGF and Ang1 are unable to promote the release of Ang2 in HUVEC [27]. To assess whether the translocation of P-selectin mediated by Ang1 or VEGF in BAEC could be a result of Ang2 release, we performed an ELISA from cell supernatant and whole cell lysate. Confluent BAEC were treated with control PBS, VEGF-A_{165} (1 nM), and Ang1 (0.01–10 nM) for 15 min. Upon stimulation with Ang1 or VEGF-A_{165}, we detected minute levels of Ang2 in the cell supernatant, which was not significantly different from basal level detected in PBS-treated BAEC (<10 pg/ml; ≈0.15 pM), whereas Ang2 was detected in whole cell lysate (500–600 pg/ml; ≈10 pM; **Fig. 4**).

Angiopoietin-mediated P-selectin translocation requires PLC-γ signal transduction

A recent study reported that VEGF-A_{165} triggers exocytosis of WPB in part through PLC-γ signal transduction [28]. We thus sought to determine the role played by PLC-γ in angiopoietin-mediated endothelial P-selectin translocation by pretreating BAEC for 15 min with pharmacological inhibitors of PLC-γ (U73122; 10 μM) and protein kinase C (PKC; Calphostin C; 100 nM) prior to stimulation with Ang1 or Ang2 (1 nM; 7.5 min). Angiopoietin-mediated endothelial P-selectin requires the activation of PLC-γ, as pretreatment with U73122 reduced Ang1- and Ang2-mediated P-selectin translocation by 56% and 88%, respectively (**Fig. 5A**). Similarly, inhibition of PKC, activated immediately downstream of PLC-γ, also reduced endothelial P-selectin translocation in BAEC treated with

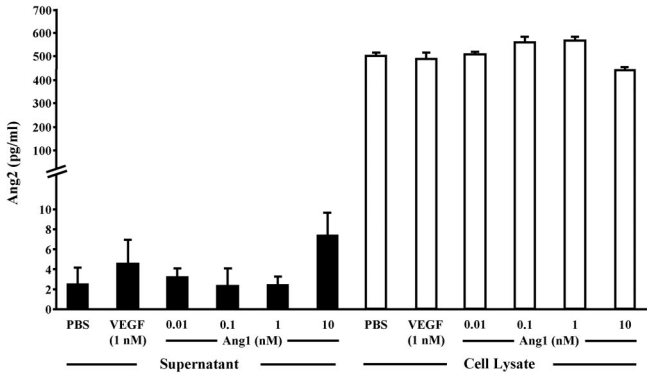

Fig. 4. Endothelial distribution of Ang2 upon stimulation with VEGF-A_{165} and Ang1. Confluent BAEC were stimulated in serum-free DMEM with VEGF-A_{165} or Ang1 for 15 min. Supernatants were collected, and Ang2 protein was quantified by ELISA. Cell membranes were gently scraped in cold PBS (pH=7.4) and disrupted by sonication, and Ang2 protein was measured in the same ELISA as the corresponding supernatants. Values are means ± SEM of experiments performed in triplicate.

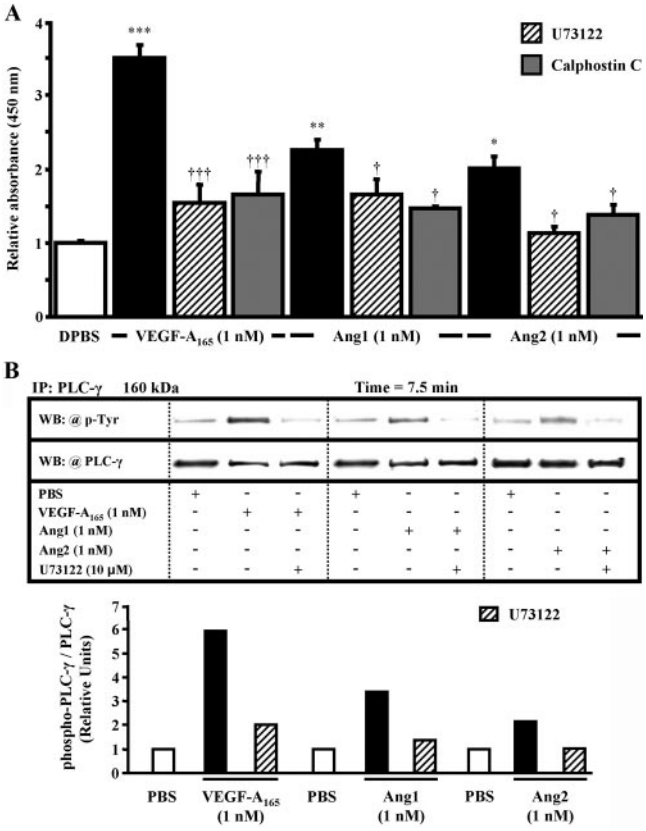

Fig. 5. Endothelial P-selectin translocation mediated by Ang1 or Ang2 requires PLC-γ and PKC signal transduction. (A) Confluent BAEC were pretreated with selective pharmacological inhibitors for PLC-γ (U73122; 10 μM) and PKC (Calphostin C; 100 nM) for 15 min prior to 7.5 min of stimulation with Ang1 or Ang2 (1 nM). BAEC were also stimulated with VEGF-A_{165} (1 nM) for 7.5 min as positive control. Endothelial P-selectin was measured by cell surface ELISA, and data are expressed as relative absorbance measured at 450 nm. ** and ***, $P < 0.01$ and $P < 0.001$, versus PBS, respectively; † and †††, $P < 0.05$ and $P < 0.001$, versus agonist, respectively. (B) The ability of Ang1 and Ang2 to activate PLC-γ was determined by Western blot analysis. Confluent BAEC were pretreated with U73122 (10 μM) 15 min prior to 7.5 min of stimulation with VEGF-A_{165}, Ang1, or Ang2 (1 nM). Values are means ± SEM of P-selectin translocation obtained from at least three independent experiments. IP, Immunoprecipitated; WB, Western blot.

Ang1 and Ang2, both by 62% (Fig. 5A). As a positive control, BAEC were pretreated with U73122 and Calphostin C prior to stimulation with VEGF-A_{165} (1 nM; 7.5 min). U73122 and Calphostin C diminished VEGF-A_{165}-mediated endothelial P-selectin translocation by 78% and 74%, respectively (Fig. 5A). Furthermore, the ability of the angiopoietins to activate PLC-γ, as well as the ability of U73122 to inhibit this activation, was demonstrated by Western blot (Fig. 5B). Pretreatment of BAEC with U73122 prior to stimulation with VEGF-A_{165}, Ang1, or Ang2 blocked PLC-γ activation (Fig. 5B).

Angiopoietins mediate endothelial P-selectin translocation independently of p38 MAPK, p42/44 MAPK, PI-3K activation, and PAF synthesis

Activation of PKC may lead to the activation of p42/44 MAPK. Furthermore, we and others [6, 8, 9, 11, 29–32] have reported that Tie2 receptor autophosphorylation following stimulation with Ang1 or Ang2 can in turn activate p38 MAPK, p42/44 MAPK,

and PI-3K/Akt intracellular signaling pathways. Pretreatment of BAEC with selective inhibitors for MAPKK (PD98059; 10 μM), p38 MAPK (SB203580; 10 μM), or PI-3K (Wortmannin; 500 nM) [33] prior to stimulation with angiopoietins did not reduce P-selectin translocation significantly (**Fig. 6A**), despite the observation that these inhibitors prevented the induction of their respective pathways (data not shown). As a positive control, pretreatment of BAEC with PD98059, SB203580, or Wortmannin reduced P-selectin translocation following 7.5 min of stimulation with VEGF-A_{165} by 55–57% (Fig. 6A).

We have reported previously about the role of endothelial PAF in VEGF-A_{165}-mediated P-selectin translocation in HUVEC [20]. To assess the contribution of endothelial PAF to angiopoietin-mediated P-selectin translocation, BAEC were pretreated with selective antagonists for intracellular (LAU 8080; 100 nM), extracellular (BN 52021; 10 μM), or intra- and extracellular (CV-3988; 10 μM) PAFR 15 min prior to stimulation with Ang1 or Ang2 (1 nM) for 7.5 min. Pretreatment with PAFR antagonists had no effect on angiopoietin-mediated P-selectin translocation (Fig. 6B). However, pretreatment of BAEC with the aforementioned PAFR antagonists (LAU 8080, BN 52021, or CV-3988) prior to stimulation with VEGF-A_{165} reduced endothelial P-selectin translocation by 59%, 39%, and, 43%, respectively (Fig. 6B).

Angiopoietin-mediated endothelial P-selectin translocation is Ca^{2+}-dependent

Angiopoietin-mediated endothelial P-selectin translocation does not appear to be regulated by the activation of p38 MAPK, p42/44 MAPK, or PI-3K intracellular signaling pathways nor by PAF. However, exocytosis of WPB and the subsequent translocation of P-selectin have been shown to be calcium (Ca^{2+})-dependent following stimulation of EC with VEGF-A_{165} [28]. To determine which Ca^{2+} pools are mobilized during angiopoietin-mediated P-selectin translocation, BAEC were pretreated with an intra- and extracellular Ca^{2+} chelator (BAPTA-AM; 10 μM) or stimulated in Ca^{2+}-free DPBS. Chelation of intra- and extracellular Ca^{2+} abrogated endothelial P-selectin translocation completely. In addition, in an extracellular Ca^{2+}-free environment, we observed that the potential release of Ca^{2+} from intracellular pools was insufficient to support endothelial P-selectin translocation (**Fig. 7A**). No significant statistical differences were observed between BAPTA-AM-treated and Ca^{2+}-free DPBS groups for each of the three growth factors studied.

To determine the role of the Ca^{2+}/CaM complex on angiopoietin-mediated endothelial P-selectin translocation, we prevented its formation with a selective inhibitor (W-7; 10 mM). The blockade of the Ca^{2+}/CaM complex completely abolished angiopoietin-mediated endothelial P-selectin translocation (Fig. 7B). Similarly, VEGF-A_{165}-mediated endothelial P-selectin translocation was also completely inhibited in response to pretreatment with W-7 (Fig. 7B).

DISCUSSION

In the present study, we demonstrate that Ang1 and Ang2 are capable of mediating Tie2 phosphorylation, resulting in a rapid and transient endothelial P-selectin translocation. In addition, we delineate that angiopoietin-mediated endothelial P-selectin translocation is Ca^{2+}-dependent through PLC-γ signal transduction. However, as opposed to VEGF-A_{165}, P-selectin translocation mediated by Ang1 or Ang2 is independent of endothelial PAF synthesis.

Ang1 and Ang2 promote Tie2 phosphorylation and endothelial P-selectin translocation

Early studies identified that Ang1 can induce a rapid activation of the Tie2 receptor [3, 4, 7, 29], whereas Ang2 was described as a natural Tie2 antagonist in EC [4]. Since then, other studies

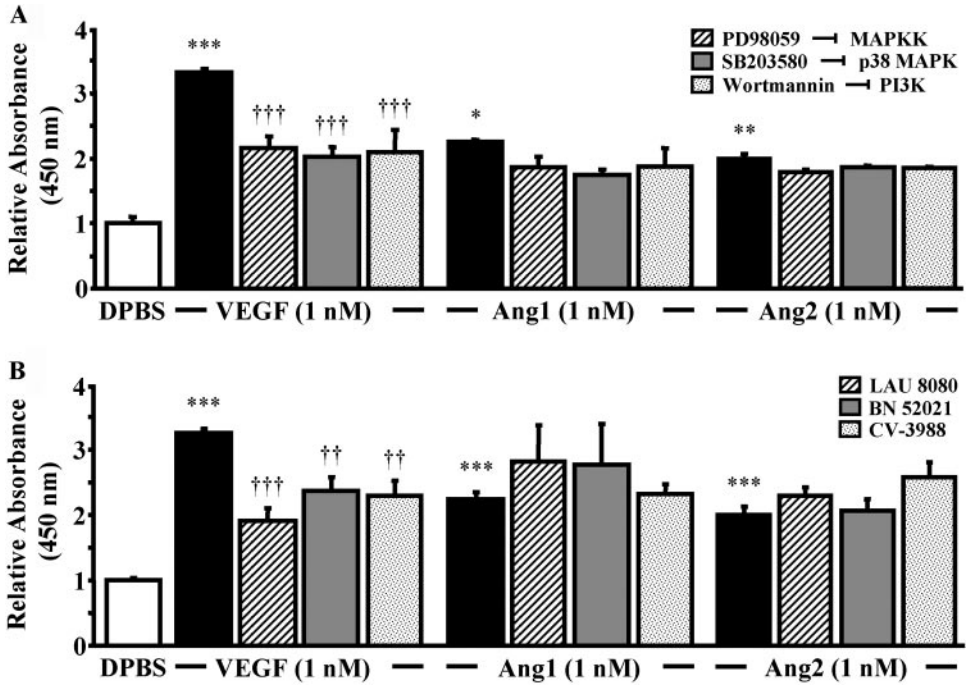

Fig. 6. p38 MAPK, p42/44, and PI-3K signaling is not required for angiopoietin-mediated endothelial P-selectin translocation. (A) Confluent BAEC were pretreated with selective inhibitors against MAPKK (PD98059; 10 μM), p38 MAPK (SB203580; 10 μM), or PI-3K (Wortmannin; 500 nM) for 15 min prior to 7.5 min of stimulation with Ang1 or Ang2 (1 nM). (B) In a separate series of experiments, confluent BAEC were pretreated with antagonists targeting the intracellular (LAU 8080; 100 nM), extracellular (BN 52021; 10 μM), or intra- and extracellular (CV-3988; 10 μM) PAF receptors (PAFR) for 15 min prior to stimulation with Ang1 or Ang2 (1 nM) for 7.5 min. BAEC were stimulated with VEGF-A_{165} (1 nM) for 7.5 min as positive control. Values are means ± SEM of P-selectin translocation obtained from at least three independent experiments. *, **, and ***, $P < 0.05$, $P < 0.01$, and $P < 0.001$, versus PBS, respectively; †† and †††, $P < 0.01$ and $P < 0.001$, versus agonist, respectively.

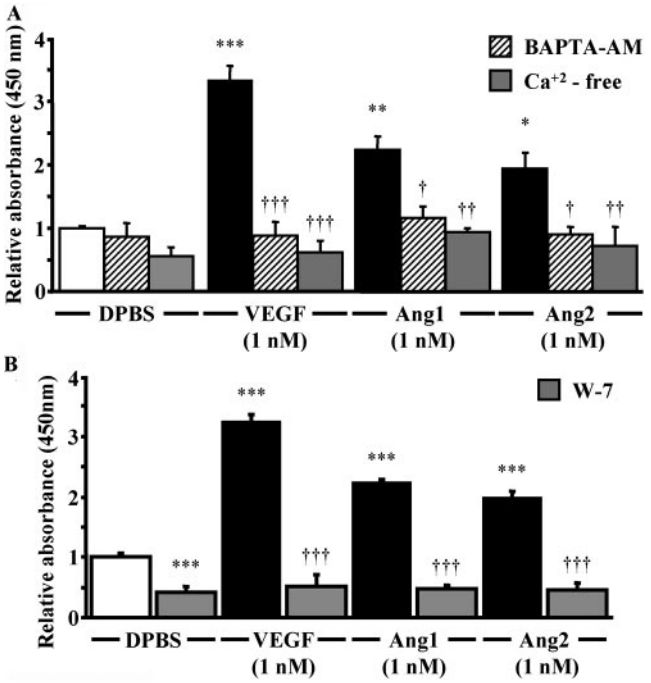

Fig. 7. Angiopoietin-mediated endothelial P-selectin requires Ca^{2+}. Confluent BAEC were pretreated with an intracellular Ca^{2+} chelator (BAPTA-AM; 10 μM) prior to stimulation with Ang1 or Ang2 (1 nM) or stimulated with angiopoietins in Ca^{+2}-free DPBS (A). In a separate series of experiments, EC were treated with a selective calmodulin (CaM) inhibitor (W-7; 10 mM) prior to treatment with Ang1 or Ang2 (1 nM). VEGF-A_{165} (1 nM; 7.5 min) is present as a positive control. Endothelial P-selectin was measured by cell surface ELISA, and data are expressed as relative absorbance measured at 450 nm. Values are means ± SEM of P-selectin translocation obtained from at least three independent experiments. *, **, and ***, $P < 0.05$, $P < 0.01$, and $P < 0.001$, versus PBS, respectively; †, ††, and †††, $P < 0.05$, $P < 0.01$, and $P < 0.001$, versus agonist, respectively.

reported that the use of high concentrations or prolonged treatment with Ang2 can induce Tie2 activation and support EC survival and EC tubule capillary-like formation [6, 7]. More recently, we and others [9–12, 15, 34] reported that Ang2 can also promote a rapid Tie2 phosphorylation (within minutes and even at lower concentrations) and mediate tubule capillary-like formation in immortomouse brain EC [15] as well as endothelial P-selectin translocation and neutrophil adhesion onto activated HUVEC [12]. In addition, we and other independent groups [12, 13, 35, 36] reported the capacity of both angiopoietins to promote a rapid Tie2 activation and chemotactic activities on neutrophils and eosinophils, and more recently, Murdoch et al. [37] reported Ang2-chemotactic activities on monocytes, without addressing the effect of Ang1. Herein, we report that both angiopoietins activate Tie2 with a similar kinetic pattern, resulting in a rapid and transient endothelial P-selectin translocation in BAEC, as demonstrated by cell surface ELISA and confocal microscopy. Our observations are consistent with reports indicating that endothelial P-selectin is translocated within minutes from WPB upon stimulation with various inflammatory mediators [38, 39]. In addition, we demonstrate that endothelial P-selectin translocation mediated by angiopoietins is concentration-dependent. Maximal P-selectin translocation was attained at 1 nM, and it is interesting that at a higher concentration (5 nM), the capacity of Ang1 and Ang2 to mediate P-selectin translocation was reduced. Similarly, both angiopoietins provided a bell-shaped chemotactic response on neutrophils [13, 35], as well as on Ang2-mediated monocyte migration [37]. We also observed this phenomenon under other experimental conditions, namely VEGF-A_{165} and angiopoietin-mediated P-selectin translocation in HUVEC, angiopoietin-mediated PAF synthesis in BAEC, as well as VEGF-A_{165}-mediated prostacyclin synthesis in BAEC [9, 12, 20, 40]. This response may be the result of an overabundance of a ligand, which can impede receptor dimerization and thus, hinder tyrosine residue autophosphorylation and a decrease in downstream signaling events [41, 42].

Angiopoietin-mediated P-selectin translocation: intracellular signaling events

In previous studies, we defined that the inflammatory properties of VEGF-A_{165} require endothelial PAF synthesis through the dual activation of PLC-γ/PKC/p42/44 MAPK and mixed lineage kinase/MAPKK (or MKK-3, -6)/p38 MAPK signaling pathways [24, 33]. We also reported that VEGF-A_{165} induces P-selectin translocation through endogenous PAF synthesis in HUVEC [20], whereas Ang1 and Ang2 can promote P-selectin translocation independently of PAF synthesis in HUVEC [12]. As Ang1 and Ang2, upon binding to Tie2, have been shown to activate p42/44 MAPK, p38 MAPK, and PI-3K intracellular signaling pathways [6, 8, 9, 29–32], and these pathways are involved in the regulation of a rapid and sustained PAF synthesis in BAEC [9], we sought to dissect the signaling pathways and the role of PAF in angiopoietin-mediated P-selectin translocation in BAEC.

In the current study, we demonstrate that the blockade of PI-3K, p42/44, and p38 MAPKs with corresponding selective inhibitors and the use of selective PAFR antagonists did not reduce angiopoietin-mediated endothelial P-selectin translocation. However, VEGF-A_{165}-mediated P-selectin translocation requires the activation of PI-3K, p38, and p42/44 MAPKs. In addition, pretreatment of BAEC with selective PAFR antagonists resulted in a reduction of VEGF-A_{165}-mediated P-selectin translocation, although not as pronounced as what we had reported previously in HUVEC [20]. Our data demonstrate that angiopoietins and VEGF-A_{165}, two different classes of tyrosine kinase receptor ligands, induce endothelial P-selectin translocation in BAEC through different mechanisms. Furthermore, the contribution of endothelial PAF to VEGF-A_{165}-mediated P-selectin translocation may account for the ability of VEGF-A_{165} to induce greater levels of P-selectin translocation than Ang1 or Ang2.

It is well established that the translocation of P-selectin and constituents of WPB require the movement of WPB from the cytoplasm and their fusion with the plasma membrane (ref. [43] for review). In addition, increased levels of cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) have been implicated in the mechanism of WPB exocytosis through the formation of a Ca^{2+}/CaM complex for a number of agonists, including thrombin and VEGF-A_{165} [28, 44]. In the current study, we observed that a treatment of BAEC with an extra- and an intracellular Ca^{2+} chelator (BAPTA-AM) or a CaM inhibitor (W-7) prior to stimulation with Ang1 or Ang2, as with VEGF-A_{165}, abrogated P-selectin translocation completely. Furthermore, P-selectin translocation was absent in cells treated with Ang1 or Ang2 in Ca^{2+}-free DPBS. Together, these data demonstrate the importance of a cytosolic Ca^{2+} increase and

Ca^{2+}/CaM complex formation in P-selectin translocation mediated by angiopoietins. In addition, in our study, we observed for VEGF-A_{165} [33], the capacity of both angiopoietins to activate PLC-γ, which in turn, can act on phosphatidylinositol 4,5 bisphosphate to produce inositol 1,4,5-trisphosphate (IP_3). Upon binding to its receptors expressed on endoplasmic reticulum (ER), IP_3 promotes release of Ca^{2+} from the ER stores (ref. [45] for review). Although we did not measure the release of intracellular Ca^{2+} per se, our data suggest that if there is a release of Ca^{2+} from intracellular stores, it is thus insufficient to support endothelial P-selectin translocation and requires extracellular Ca^{2+} entrance. Additional experiments would be required to better delineate the capacity of angiopoietins to modulate the release of Ca^{2+} from intracellular pools.

As P-selectin and Ang2 are stored separately in endothelial WPB [27] and that VEGF, Ang1, and Ang2 are capable of promoting endothelial P-selectin translocation in HUVEC [12, 20], we performed an ELISA to assess the capacity of VEGF and Ang1 to mediate Ang2 release by BAEC. In both cases, we observed that neither Ang1 nor VEGF was capable of promoting Ang2 release. Our data are in line with a previous study reporting that Ang1 and VEGF are unable to promote Ang2 release in HUVEC [27], thus suggesting that Ang1 and VEGF-mediated P-selectin translocation is independent of Ang2 secretion in HUVEC and BAEC.

Previous studies reported that under in vitro conditions, Ang1 possesses anti-inflammatory properties. For instance, prolonged treatment with Ang1 reduces VEGF-induced leukocyte adhesion onto HUVEC and E-selectin expression [46] and thrombin-mediated neutrophil adhesion onto EC [47]. Upon initial review, these results may appear contradictory to what we report herein as well as what we have demonstrated previously [12]. However, both of the above studies [46, 47] were performed for extended periods of time well in excess of the timeframe of P-selectin activity, and to this regard, it is to remember that P-selectin is constitutively stored within WPB and that the stimulation of EC by various proinflammatory mediators can lead to the fusion of WPB to cell surface membrane and P-selectin translocation. Based on the current and recent observations, namely, on the capacity of Ang1 and Ang2 to promote PAF synthesis, endothelial P-selectin translocation in two different EC types, neutrophil adhesion, potentiating neutrophil migration mediated by a proinflammatory mediator (IL-8) [9, 12, 13], and that similar observations were made by independent groups regarding the capacity of Ang1 and Ang2 to promote the migration of eosinophils and neutrophils [35, 36], as well as monocytes (at least under Ang2 stimulation) [37], we are led to suggest that both angiopoietins can behave, under specific in vitro conditions and in various cell types, as proinflammatory mediators.

Under in vivo conditions, there is mounting evidence demonstrating that both angiopoietins can promote agonistic Tie2 activities and that in the function of the experimental model, Ang1 and Ang2 can modulate proinflammatory activities. Previous studies, for instance, reported that genetic overexpression or overexpression following infection with adenovirus carrying the Ang1 gene did protect the vasculature from VEGF and irritant-induced leakage in mice [5, 48], whereas other studies reported that Ang2 can promote vascular permeability increase [49, 50] and potentiate TNF-α-mediated proinflammatory activities. However, this paradigm, where Ang1 and Ang2 can serve as anti- and proinflammatory mediators, respectively, has been challenged recently by two independent studies. Roviezzo et al. [14] demonstrated under in vivo conditions (mouse paw edema model) that an intradermal injection of Ang2 induced rapid edema formation. However, in their study, they also observed that the administration of Ang1 did not inhibit VEGF-induced vascular permeability but delayed edema resolution, prompting the authors to suggest that Ang1 is not a universal inhibitor of EC permeability as thought previously [14]. On one hand, the group of Daly et al. [34] reported that upon an i.v. injection of Ang1 or Ang2, both were capable to promote a rapid Tie2 phosphorylation in mouse heart tissue; however, an infection of mice with adenoviruses encoding Ang1 or Ang2 significantly reduced vascular permeability mediated by mustard oil. For the latter study, the authors speculated that the Ang2 anti-inflammatory activity observed as opposed to the previous reports [14, 49, 50] could result from different methods of Ang2 administration or from analysis of Ang2 actions in different tissues.

Taken together, our observations and those presented above demonstrate that the initial conclusions pertaining to the respective role of Ang1 and Ang2 about the modulation of inflammatory events were perhaps overly simplistic.

In summary, we demonstrate that in BAEC and under our experimental conditions, Ang1 and Ang2 are capable of mediating agonistic activities, namely by inducing a rapid and transient translocation of P-selectin, which is dependent on extracellular Ca^{2+} entrance and PLC-γ activation. However, contrary to VEGF-A_{165}, angiopoietins do not require endogenous PAF synthesis to support endothelial P-selectin translocation.

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