Available online at www.sciencedirect.com
ScienceDirect
Cellular Signalling 18 (2006) 1947–1957
CELLULAR SIGNALLING
www.elsevier.com/locate/cellsig

Angiopoietins-1 and -2 are both capable of mediating endothelial PAF synthesis: Intracellular signalling pathways

Ricardo Maliba, Stéphanie Lapointe, Paul-Eduard Neagoe, Alexandre Brkovic, Martin G. Sirois *

Montreal Heart Institute and Department of Pharmacology, Université de Montréal, Montreal, QC, Canada

Received 9 November 2005; received in revised form 27 February 2006; accepted 28 February 2006
Available online 17 April 2006

Abstract

Vascular endothelial growth factor (VEGF) is the only angiogenic growth factor capable of inducing an inflammatory response and we have recently demonstrated that its inflammatory effect is mediated by the endothelial synthesis of platelet-activating factor (PAF). Recently discovered, Ang1 and Ang2, upon binding to Tie2 receptor, modulate vascular permeability and integrity, contributing to angiogenesis. Ang1 was initially identified as a Tie2 agonist whereas Ang2 can behave as a context-dependent Tie2 agonist or antagonist. We sought to determine if Ang1 and/or Ang2 could modulate PAF synthesis in bovine aortic endothelial cells (BAEC) and if so, through which intracellular signalling pathways. Herein, we report that Ang1 and Ang2 (1 nM) are both capable of mediating a rapid Tie2 phosphorylation and a rapid, progressive and sustained endothelial PAF synthesis maximal within 4 h (1695% and 851% increase, respectively). Angiopoietin-mediated endothelial PAF synthesis requires the activation of the p38 and p42/44 MAPKs, PI3K intracellular signalling pathways, and a secreted phospholipase A_2 (sPLA$_2$-V). Furthermore, angiopoietin-mediated PAF synthesis is partly driven by a relocalization of endogenous VEGF to the cell surface membrane. Our results demonstrate that the angiopoietins constitute another class of angiogenic factors capable of mediating PAF synthesis which may contribute to proinflammatory activities.

Keywords: Angiopoietins; Tie2 receptor; Platelet-activating factor; Inflammation

1. Introduction

Angiogenesis plays a critical role in several pathological conditions, namely atherosclerosis, proliferative retinopathies, and tumor growth [1]. Previous studies established the contribution of vascular endothelial growth factor (VEGF-A_{165}) and the cell signalling mechanisms by which it leads to angiogenesis [1]. Namely, it has been reported that inflammation precedes and accompanies pathological angiogenesis as evidenced by increased vascular permeability, monocyte/macrophage and neutrophil recruitment at angiogenic sites [2]. During inflammatory processes, newly formed vessels supply inflamed tissue with oxygen and nutrients as well as facilitate the transport of inflammatory cells. Recently, we have shown that VEGF-A_{165} increases vascular permeability through the synthesis of a potent inflammatory mediator, platelet-activating factor (PAF) by endothelial cells (EC) [3]. VEGF-mediated endothelial PAF synthesis occurs via a remodeling pathway in which membrane phospholipids are converted by a phospholipase A_2 (sPLA$_2$-V) into lyso-PAF which is in turn acetylated into PAF by acetylCoA:lyso-PAF acetyltransferase (lyso-PAF AT) [4]. Furthermore, we have recently demonstrated that in bovine aortic endothelial cells (BAEC), VEGF-A_{165} activation of both p38 and p42/44 mitogen-activated protein kinases (MAPK) are crucial to VEGF-mediated endothelial PAF synthesis whereas phosphatidyl inositol-3-phosphate kinase (PI3K) activation is not required [5]. Moreover, newly synthesized PAF is essential for VEGF-A_{165}-mediated endothelial P-selectin translocation and neutrophil adhesion onto activated EC [3,6,7], essential events in the induction of acute inflammatory processes.

Recently, a new class of angiogenic factors, angiopoietins (Ang1 and Ang2), was defined as ligands for the tyrosine kinase receptor Tie2 [8,9] to which they bind with similar specificity and affinity [8,9]. Ang1 has been characterized as a Tie2 agonist,

* Corresponding author. Montreal Heart Institute 5000 Belanger Street Montreal, QC, Canada, H1T 1C8. Tel.: +1 514 3763330x3583; fax: + 514 3761355.
E-mail address: martin.sirois@icm-mhi.org (M.G. Sirois).

0898-6568/$ - see front matter.
doi:10.1016/j.cellsig.2006.02.015

having the capacity to stabilize and promote the maturation of unstable vessels in the presence of VEGF-A_{165} [10]. On the other hand, Ang2 was initially described as a natural endogenous Tie2 antagonist, thereby destabilizing existing vessels prior to VEGF-A_{165}-induced angiogenic sprouting [9]. However, recent findings have shown that Ang2 may, under certain circumstances, induce Tie2 phosphorylation and biological activities such as EC migration, and in vitro tubule capillary-like formation [11,12]. In addition, we recently demonstrated that both angiopoietins can promote endothelial P-selectin translocation, directly activate neutrophils through Tie2 signalling as well as modulate PAF synthesis and β_2-integrin functional upregulation thereby promoting the acute recruitment of leukocytes and conferring a proinflammatory capacity to angiopoietins [13].

Based on our previous observations with regards to VEGF-A_{165} and on the potential capacity of angiopoietins at regulating vascular integrity, we sought to investigate whether Ang1 and/or Ang2 modulate endothelial PAF synthesis and if so, to define the intracellular signalling pathways.

2. Material and methods

2.1. Cell culture

Bovine aortic endothelial cells (BAEC) were isolated from freshly harvested aortas, cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Pickering ON) containing 5% fetal bovine serum (FBS; Medicorp Inc., Montreal, QC) and antibiotics (Sigma, St. Louis, MO). BAEC were characterized as previously described and used between passages 3 and 7 [3,14].

2.2. Western blot analysis of Tie2 and VEGFR-2 phosphorylation

Confluent BAEC were serum-starved in DMEM with antibiotics overnight, rinsed with Hank's balanced salt solution (HBSS; Life Technologies, Burlington, ON), then stimulated in a solution of HBSS/HEPES (10 mM, pH 7.4), bovine serum albumin (BSA; 1 mg/mL; Sigma), and $CaCl_2$ (10 mM). Cells were placed on ice for 30 min then stimulated with Dulbecco's phosphate-buffered saline (PBS), Ang1, or Ang2 (1 nM; R&D Systems, Minneapolis, MN) at 37 °C for up to 2 h. In another set of experiments, BAEC were pretreated with selective inhibitors of VEGFR-1 and VEGFR-2 (VTK; 10 μM; IC_{50}=2.0 and 0.1 μM respectively) [15,16], or VEGFR-2 (SU1498; 5 μM; IC_{50}=0.7 μM) [7,16,17] (Calbiochem, La Jolla, CA), 15 min prior to stimulation with Ang1 or Ang2 (1 nM). In a third set of experiments, we assessed the capacity of angiopoietins (Ang1 and Ang2) to transactivate VEGFR-2 in function of time. Cells were solubilized with lysis buffer, scraped, and protein concentration determined by Bradford assay. Cell lysates were immunoprecipitated with rabbit polyclonal anti-mouse Tie2 IgG or with anti-mouse VEGFR-2 IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and separated by SDS–PAGE. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and probed with mouse monoclonal anti-phosphotyrosine IgG (clone 4G10, 1:1000 dilution; Upstate Biotechnology Inc., Lake Placid, NY). Membranes were stripped using Re-Blot Plus Strong stripping solution (Chemicon International, Temecula, CA) and reprobed with rabbit polyclonal anti-mouse Tie2 IgG or VEGFR-2 IgG (1:1000 dilution; Santa Cruz Biotechnology). Bands were visualized using LumiGlo™ (New England Biolabs, Pickering, ON). The density of the bands was determined using Quantity One software (Bio-Rad, Mississauga, ON) [7].

2.3. Western blot analysis of p38, p42/44, and Akt activation by angiopoietins

Confluent BAEC were serum-starved overnight, rinsed, and stimulated with Ang1 or Ang2 (1 nM) for various time durations. In another series of experiments, BAEC were pretreated with either a p38 MAPK inhibitor (SB203580, 10 μM), MAPK kinase (MAPKK) inhibitor (PD98059, 10 μM), or inhibitors of the PI3K/Akt pathway (LY294002, 5 μM; 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., Rocky Hill, NJ) for 7.5 min after pretreatment with the aforementioned pathway inhibitors. Cell lysates were separated by SDS–PAGE and proteins transferred onto a PVDF membrane. Activation of p38, p42/44 and Akt was determined by probing membranes with antibodies for their respective phosphorylated forms (1:1000 dilution; New England Biolabs). Membranes were subsequently stripped and reprobed to visualize corresponding total protein expression.

2.4. Measurement of PAF synthesis

Confluent BAEC were rinsed, then stimulated in HBSS/HEPES containing $CaCl_2$ (10 mM) and [^{3}H]-acetate (25 μCi) (New England Nuclear, Boston, MA) with angiopoietins (Ang1 or Ang2; 0.1 to 10 nM) or VEGF-A_{165} (1 nM) for 7.5 to 360 min. In another series of experiments, BAEC were pretreated with VTK (10 μM), SU1498 (5 μM), SB203580 (10 μM), PD98059 (10 μM), LY294002 (5 μM) or Wortmannin (500 nM) prior to stimulation with Ang1, Ang2, or VEGF-A_{165}. BAEC were also pretreated with either a selective cPLA$_2$ and iPLA$_2$ inhibitor ($AACOCF_3$; 10 μM; Calbiochem), a non-specific sPLA$_2$ inhibitor (scalaradial; 10 μM; Calbiochem), or a selective sPLA$_2$-V inhibitor (LY311727; 100 μM; kindly provided by Dr. Jerome Fleisch, Lilly Research Laboratories, Indianapolis, IN) for 15 min prior to stimulation with Ang1 or Ang2 (1 nM) for 240 min or VEGF-A_{165} for 15 min. The reaction was halted by addition of acidified methanol, polar lipids isolated, evaporated under N_2 gas, and purified by HPLC as described previously [3–5]. Fractions corresponding to [^{3}H]-PAF were quantified with a β-counter. The authenticity of synthesized PAF was confirmed by an identical elution pattern to standard [^{3}H]-PAF (New England Nuclear) [3,14].

2.5. VEGF ELISA

VEGF protein in BAEC supernatant and whole cell extract was quantified using a commercial ELISA kit (PeproTech Inc.). Confluent cells grown in 6-well plates were serum-starved overnight in DMEM containing antibiotics prior to stimulation with Ang1 or Ang2 (1 nM) in HBSS–HEPES containing $CaCl_2$ (10 mM) for various time periods. Upon stimulation, cell supernatant was collected, the cells scraped, and gently sonicated in PBS (pH 7.4) in ice. The ELISA protocol was carried out according to the manufacturer's instructions.

2.6. Confocal microscopy: image acquisition, deconvolution and image rendering

BAEC were grown to confluence on glass coverslips coated with 1.5% gelatin, serum-starved overnight, rinsed, and incubated with rabbit polyclonal anti-human VEGF IgG (1:100 dilution; Santa Cruz Biotechnology) in the presence of Ang1 or Ang2 (1 nM; 7.5 to 240 min) in serum-free DMEM. Following stimulation, the cells were rinsed and fixed with a 1% paraformaldehyde-PBS solution for 20 min. Nonspecific binding of primary antibodies was prevented by preincubating live BAEC with 4% serum from the species used to raise the secondary antibodies. Cells were rinsed and incubated with donkey anti-rabbit Alexa 555 conjugated IgG (1:400 dilution; Molecular Probes, Eugene, OR) for 90 min. Glass coverslips were mounted using 1,4-diazabicyclo-2-2-2octane (DABCO/glycerol (1:1) solution. BAEC were observed on a Zeiss Axiovert 100 M microscope equipped with a 63X/1.4 Plan-Apochromat oil objective lens (Zeiss, Oberkochen, Germany) adapted with an LSM 510 confocal system and saved as LSM files. Donkey anti-rabbit conjugated to Alexa 555 IgG was visualized using a 543 nm Helium–Neon laser. Voxel size is 143×143×160 nm (*X*, *Y*, *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* stacks and added to the MLE algorithm. Point spread function (PSF) was derived from *Z* stacks of 15 fluorescent (540–560 nm) beads of 170 nm in diameter (Invitrogen). PSF was acquired the same way as the images of interest. Deconvolutions were applied until reaching 0.01% quality change threshold (QCT) between iterations. Deconvolved *Z* stacks were saved in Tiff file format series. Transparent projections were produced using the projection tool of

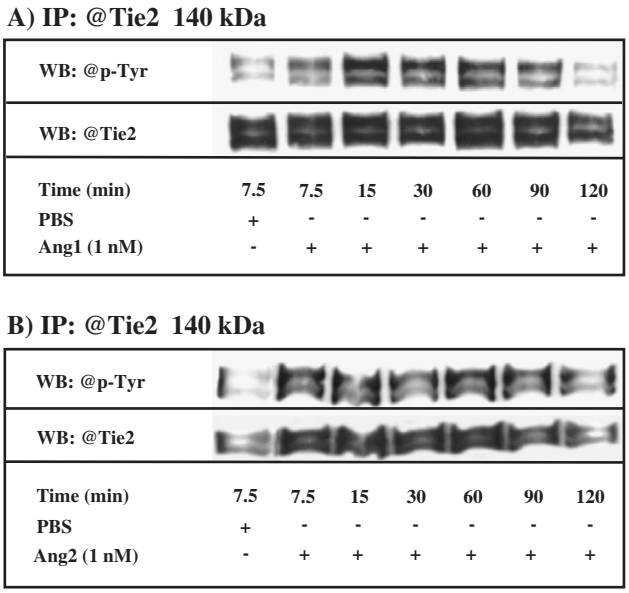

Fig. 1. Activation and expression of Tie2 in BAEC. Confluent BAEC were treated with Ang1 (A), or Ang2 (B) for up to 2 h. Cell lysates were prepared and immunoprecipitated (IP) with rabbit polyclonal anti-mouse Tie2 IgG from 500 μg of lysate. Following resolution by SDS–PAGE and transfer, PVDF membranes were probed with mouse monoclonal antiphosphotyrosine IgG and immunoreactive bands were visualized by chemiluminescence. Membranes were subsequently stripped using ReBlot Plus Strong stripping solution and Tie2 protein expression was determined following incubation with rabbit polyclonal anti-mouse Tie2 IgG. IP designates immunoprecipitation and WB represents Western blot.

the LSM 510 software. VEGF levels at the cell surface membrane were assessed by quantifying the summation of voxel intensity of the deconvolved *Z* stacks volume using the Huygens Pro 2.6.5a software. The relative intensity (RI) of VEGF at the cell surface membrane was set at 1 for the PBS-control treated cells.

2.7. Statistical analysis

Data are mean+SEM. Comparisons were made by analysis of variance followed by a Bonferroni *t*-test. Data were considered significantly different if values of $p<0.05$ were observed.

3. Results

3.1. Activation of Tie2 receptor by angiopoietins

We first 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 and transient phosphorylation of Tie2, which was maximal within 15 min, and corresponding to a 21-fold increase over PBS-treated cells (Fig. 1A). Treatment with Ang2 (1 nM) also induced a rapid and transient activation of Tie2 leading to an 8-fold increase in phosphorylation within 7.5 min (Fig. 1B).

3.2. Activation of p38 MAPK, p42/44 MAPK, and Akt by angiopoietins

Previous studies reported that Ang1 is capable of activating p38 and p42/44 MAPKs [18] as well as the PI3K/Akt signal transduction pathways [19]. Prior to our study, little was known with regards to potential intracellular events following the activation of Tie2 by Ang2. Herein, we demonstrate that Ang2, like Ang1, can activate p42/44 and p38 MAPK as well as PI3K in a time-dependent manner (Fig. 2). In our study, stimulation of BAEC with Ang1 (1 nM) activates p42/44 MAPK and Akt in a time-dependent manner (Fig. 2A and E) with maximal effects at 7.5 min maintained through 30 min of stimulation. Treatment with Ang1 induces a rapid and transient activation of p38 MAPK with a maximal phosphorylation at 7.5 min (Fig. 2C). Similarly, Ang2 (1 nM) activates all three pathways but with slight variations in its kinetics. Firstly, maximal activation of p42/44 MAPK occurs within 10 min of stimulation (Fig. 2B) but as with Ang1, this activation is maintained 30 min post-stimulation. Secondly, activation of PI3K/Akt by Ang2 is delayed compared to Ang1 and not sustained (20 min versus 7.5 min; Fig. 2F). Ang2 activation of p38 MAPK produces a pattern similar to what was observed with Ang1 (Fig. 2D). As a positive control, BAEC were also stimulated with VEGF-A_{165} (1 nM; 7.5 min).

3.3. Regulation of PAF synthesis by Ang1 and Ang2

We previously reported that VEGF-A_{165} induces a rapid and transient (within 15 min) endothelial PAF synthesis [3,14]. Therefore, we assessed the capacity of angiopoietins to mediate endothelial PAF synthesis. The induction of PAF synthesis by the angiopoietins was very rapid (significant increase within 7.5 min), maximal at 4 h and sustained for at least 6 h post-treatment (Fig. 3A–B). Angiopoietin-mediated endothelial PAF synthesis is characterized by a biphasic response profile. An initial rapid and moderate synthesis is observed from 7.5 to 30 min followed by a "burst" phase culminating at 4 h. The peak values of PAF synthesis mediated by Ang1 and Ang2 correspond to a 1695% and 851% increase, respectively, compared to PBS-treated cells. Basal levels of PAF synthesis in PBS-treated cells did not change significantly throughout the time course of the experiments (data not shown). In addition, VEGF-A_{165} (1 nM) was used as positive control and induced maximal PAF synthesis within 15 min (788% increase over PBS values) and was degraded within 30 min, as previously described [3,14]. We also assessed the potential of Ang1 and Ang2 to mediate endothelial PAF synthesis in a concentration-dependent manner. Cells were treated with Ang1 or Ang2 (0.1 to 1 nM) at an intermediate time period (2 h) to ensure that we were not reaching a saturation plateau of PAF synthesis. PAF synthesis mediated by Ang1 at 0.1 nM was almost as potent as at 1 nM whereas Ang2 at 0.1 nM did not significantly increase PAF synthesis but at 1 nM, Ang2 had an equivalent agonistic activity, compared to Ang1 at mediating endothelial PAF synthesis after 2 h (Fig. 3C). Interestingly, at a higher concentration (10 nM), both Ang1 and Ang2 almost completely lost (86% and 75%, respectively) their capacity of mediating PAF synthesis in comparison to 1 nM (Fig. 3C).

3.4. Role of endogenous VEGF in angiopoietin-mediated PAF synthesis

Since the stimulation of BAEC with VEGF-A_{165} induces PAF synthesis and that EC express VEGF [20–23], we hypothesized

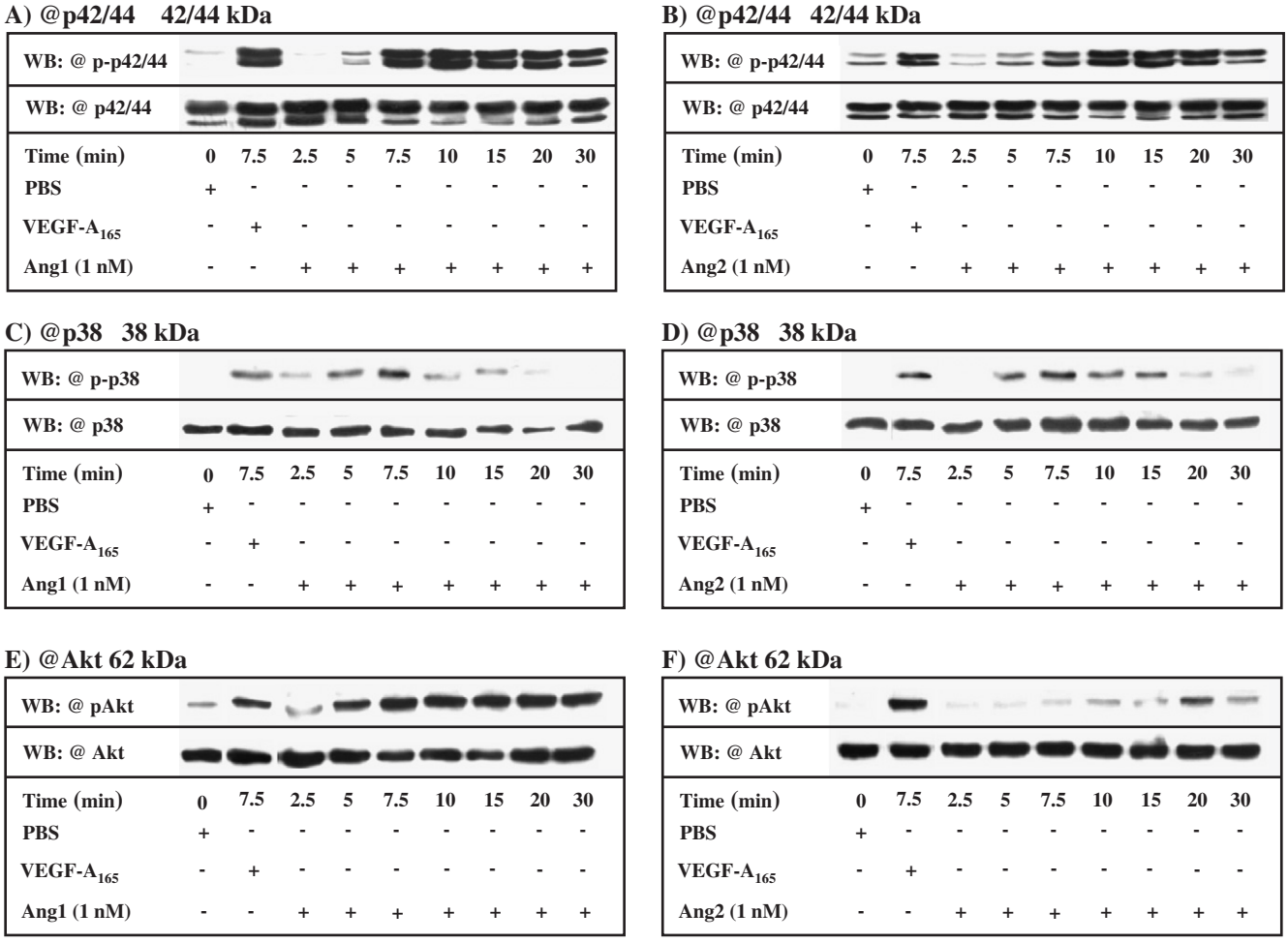

Fig. 2. Ang1 and Ang2 activate p38 MAPK, p42/44 MAPK, and PI3K pathways. Confluent BAEC were stimulated with Ang1, or Ang2 (1 nM) for up to 30 min. BAEC were also stimulated with VEGF-A_{165} for 7.5 min as positive control. Cell lysates equivalent to 100 μg total proteins were loaded in each lane. Signalling pathway activation was determined by probing PVDF membranes with antibodies for the phosphorylated form of p42/44 MAPK, p38 MAPK, or PI3K/Akt. Ang1 and Ang2 activate p42/44 MAPK (A and B, respectively), p38 MAPK (C and D, respectively), and PI3K/Akt (E and F, respectively). Membranes were then stripped and corresponding protein expression determined.

that VEGF may contribute to angiopoietin-mediated endothelial PAF synthesis. Therefore, we pretreated BAEC with inhibitors of both VEGFR-1 and VEGFR-2 (VTK; 10 μM) or VEGFR-2 (SU1498; 5 μM) for 15 min prior to stimulation with Ang1 or Ang2 (1 nM) for 4 h. Inhibition of VEGFR-1 and VEGFR-2 with VTK prompted a 51% and 43% decrease in PAF synthesis mediated by Ang1 and Ang2, respectively (Fig. 4). When only VEGFR-2 activity was inhibited, Ang1 and Ang2-mediated PAF synthesis was diminished by 42% and 26%, respectively (Fig. 4). As a positive control, the above inhibitors were added individually prior to VEGF-A_{165} stimulation and completely abrogated VEGF-A_{165}-mediated PAF synthesis at 15 min (Fig. 4). In addition, to assess that these inhibitors of VEGF receptors were not interfering with Tie2 phosphorylation mediated by angiopoietins, we pretreated BAEC with VTK or SU1498 15 min prior to stimulation with angiopoietins (1 nM) for 7.5 min. Such pretreatment with the aforementioned inhibitors did not alter angiopoietin-mediated Tie2 phosphorylation (data not shown).

We then investigated whether VEGF was released from BAEC to promote its autocrine activity on PAF synthesis. We performed an ELISA assay and detected negligible amounts of VEGF in the supernatant of BAEC treated with Ang1 or Ang2 from 15 min to 6 h, whereas most endogenous VEGF was quantified from BAEC lysates (Fig. 5). Confocal microscopy was then employed to visualize the distribution of VEGF within BAEC. Labeling live cells with primary antibodies targeting VEGF prior to stimulation allowed us to observe the relocalization of endogenous VEGF to the cell surface membrane. In control PBS-treated cells, the relative intensity (RI) of VEGF protein detection on the cell surface membrane was set to 1 (Fig. 6A), and was slightly higher than the negative control in which PBS-treated cells were incubated with isotypic rabbit IgG instead of primary VEGF IgG (RI = 0.9; Fig. 6B). Treatment with Ang1 (1 nM) for 7.5 min resulted in a marked redistribution of VEGF on the cell surface membrane (RI = 10.2; Fig. 6C), which remained noticeable up to 4 h post-stimulation (RI = 1.97; Fig. 6D). Treatment of BAEC with Ang2 (1 nM) also induced an acute but less intense relocalization of endogenous VEGF within 7.5 min (RI = 2.81; Fig. 6E), but was sustained up to 4 h (RI = 3.56; Fig. 6F).

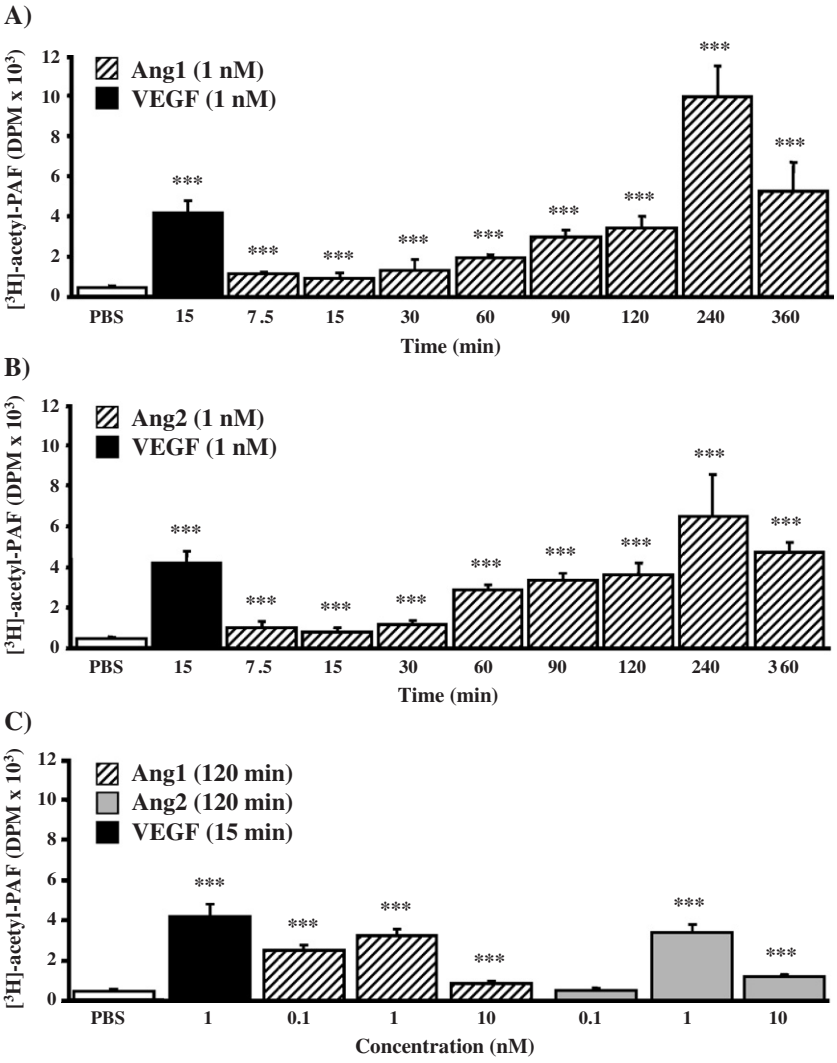

Fig. 3. Ang1 and Ang2 are both capable of inducing endothelial PAF synthesis in a time and concentration-dependent manner. Confluent BAEC were stimulated with Ang1 or Ang2 (1 nM) for time periods ranging from 7.5 to 360 min in the presence of [^{3}H]-acetate. As positive control BAEC were treated with VEGF-A_{165} for 15 min. Ang1 (A) and Ang2 (B) mediate endothelial PAF synthesis with maximal values observed following 4 h of stimulation. Maximal angiopoietin-mediated endothelial PAF synthesis is observed when Ang1 and Ang2 are used at a concentration of 1 nM (C). Data are expressed as thousands (10^3) disintegrations per minute (DPM) and represent the incorporation of tritiated acetate; [^{3}H]-acetate into lyso-PAF. Values are means±SEM of at least 12 experiments. ***$p<0.001$ vs. PBS.

Since angiopoietins mediate VEGF relocalization to the cell surface membrane of endothelial cells, and that VEGF-A_{165} mediates PAF synthesis through VEGFR-2 activation, we then sought to assess whether angiopoietins can promote VEGFR-2 transactivation. We observed that a treatment with Ang1 or Ang2 (1 nM) mediated a rapid and transient VEGFR-2 phosphorylation, which was maximal within 15 and 30 min (6.3 and 9.2-fold increase), respectively (Fig. 7A and B). Treatment with VEGF-A_{165} (1 nM) for 7.5 min was used as positive control (Fig. 7C).

3.5. Contribution of p38 MAPK, p42/44 MAPK, and PI3K to angiopoietin-mediated PAF synthesis

We recently reported that endothelial VEGF-A_{165}-mediated PAF synthesis by BAEC involves p38 and p42/44 MAPKs activation whereas PI3K activation does not lead to PAF synthesis [5]. Subsequently, in order to determine the intracellular pathways by which the angiopoietins promote EC PAF synthesis, we pretreated BAEC with selective inhibitors of the corresponding signalling pathways. Pretreatment of BAEC with a MAPKK inhibitor (PD98059; 10 μM), a p38 MAPK inhibitor (SB203580; 10 μM), or two unrelated selective PI3K inhibitors (LY294002; 5 μM and Wortmannin; 500 nM) 15 min prior to treatment with Ang1 (1 nM) for 4 h, decreased EC PAF synthesis by 65%, 93%, 93%, and 100%, respectively (Fig. 8A). Similarly, Ang2-mediated EC PAF synthesis at 4 h was reduced by 73% to 100% following pretreatment with the aforementioned inhibitors (Fig. 8A). As positive control, these inhibitors were used prior to VEGF-A_{165} stimulation and PAF synthesis was completely blocked following pretreatment with PD98059 and SB203580 whereas pretreatment with PI3K inhibitors did not reduce PAF synthesis (data not shown). We also performed Western blot analyses to confirm that the selective inhibitors at corresponding

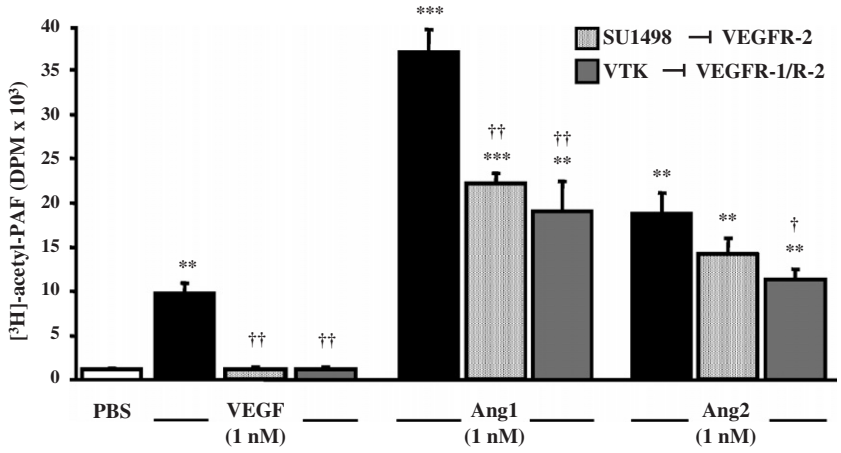

Fig. 4. Pretreatment with VEGF receptor (VEGFR) inhibitors attenuates angiopoietin-mediated endothelial PA synthesis at 4 h. Confluent BAEC were pretreated with a selective VEGFR-2 inhibitor (SU1498; 5 μM) or a VEGFR-1/R-2 inhibitor (VTK; 10 μM) for 15 min prior to stimulation with Ang1 or Ang2 (1 nM) for 4 h in the presence of [^{3}H]-acetate. Values are means±SEM of at least three experiments. $^{**}p<0.01$ and $^{***}p<0.001$ vs. PBS; †$p<0.05$ and ††$p<0.01$ vs. agonist.

concentrations prevented the phosphorylation of p42/44 MAPK, p38 MAPK and PI3K (data not shown).

3.6. The effect of angiopoietins on endothelial PAF synthesis is attenuated by sPLA$_2$ inhibitors

We have previously demonstrated the role of sPLA$_2$-V in VEGF-mediated EC PAF synthesis [4] and therefore sought to determine which phospholipase A$_2$ is implicated in angiopoietin-mediated EC PAF synthesis. We observed that inhibition of cPLA$_2$ and iPLA$_2$ using AACOCF$_3$ (10 μM) failed to reduce angiopoietin-mediated EC PAF synthesis (Fig. 8B). However, pretreatment with a broad-range sPLA$_2$ inhibitor, scalaradial (10 μM), inhibited Ang1 and Ang2-mediated EC PAF synthesis by 57% and 51%, respectively (Fig. 8B). We also treated BAEC with LY311727 (100 μM), an inhibitor of sPLA$_2$-V 15 min prior to stimulation with Ang1 or Ang2 (1 nM; 4 h) and observed a reduction of 43% and 55% in PAF synthesis, respectively (Fig. 8B). In addition, we performed a positive control study in which the corresponding inhibitors were added prior to VEGF-A$_{165}$ wherein we observed that EC PAF synthesis was almost totally abrogated (97% inhibition) following pretreatment with both non-specific (scalaradial) and specific (LY311727) sPLA$_2$-V inhibitors whereas the inhibition of cPLA$_2$ (AACOCF$_3$; 10 μM) did not attenuate endothelial PAF synthesis (data not shown).

4. Discussion

In the present study, we observed that Ang1 and Ang2 are both capable of mediating a rapid Tie2 phosphorylation, as well as a rapid, progressive and sustained endothelial PAF synthesis. This angiopoietin-mediated PAF synthesis, maximal at 240 min is mediated in part by a relocalization of endogenous VEGF to the cell membrane and through the activation of the p38 MAPK, p42/44 MAPK, and PI3K/Akt intracellular signalling pathways acting on a secreted phospholipase A$_2$ (sPLA$_2$-V).

4.1. Ang1 and Ang2 both act as Tie2 agonists

Our data showed that Ang1 and Ang2 are both capable of mediating a rapid and transient phosphorylation of Tie2 receptor, which is in agreement with previous reports [8,11,12,18,24]. One of the major characteristics of our study is that Ang1 and

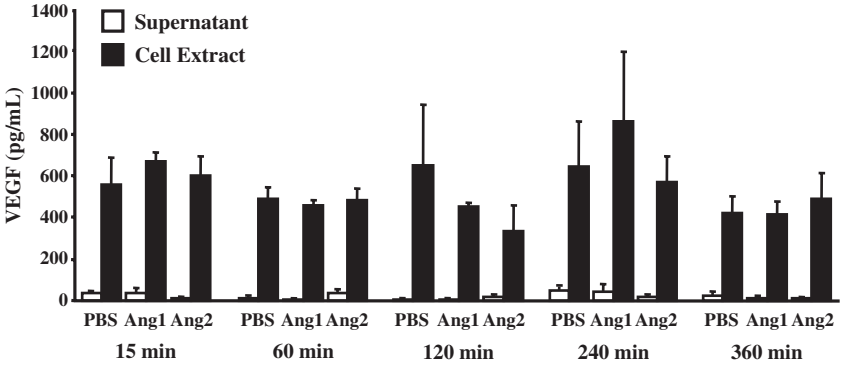

Fig. 5. Endothelial distribution of VEGF upon stimulation with angiopoietins. Confluent BAEC were stimulated in serum-free DMEM with Ang1 or Ang2 (1 nM) for up to 6 h. Supernatants were collected, concentrated and VEGF protein quantified by sandwich ELISA. Cell membranes were gently scraped in cold PBS (pH=7.4), disrupted by sonication, and VEGF protein measured in the same ELISA as the corresponding supernatants. Values are means±SEM corresponding to three experiments.

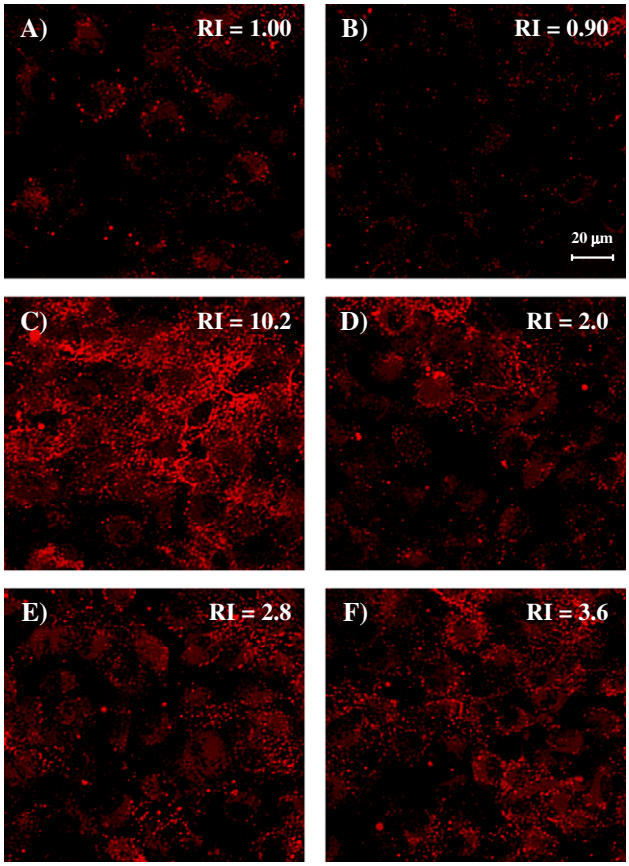

Fig. 6. Treatment of BAEC with angiopoietins induces a relocalization of endogenous VEGF to the cell surface membrane. Subconfluent BAEC were grown on gelatin-coated glass slides, rinsed with PBS, left untreated (A and B) or treated with Ang1 or Ang2 (both 1 nM) for 7.5 min (C and E, respectively) or 4 h (D and F, respectively). Prior to angiopoietin stimulation, cells were incubated with rabbit polyclonal anti-human VEGF antibody (A, C–F) or with isotypic IgG (B) and then fixed with 1% paraformaldehyde. The presence of VEGF on the cell surface membrane was observed by confocal microscopy. Relative intensity (RI) for each image represents the summation of voxel intensities compared to that of PBS which was normalized to 1. Bar represents 20 µm.

Ang2 at 1 nM were able to phosphorylate Tie2 in a manner corresponding to 21- and 8-fold increase compared to PBS-treated cells, and at such concentration, both had their maximal agonistic effect on PAF synthesis, increasing it by 1695% and 851%, respectively. Our results suggest that Ang2 might serve as a partial Tie2 agonist on PAF synthesis, and are in agreement with a recent study demonstrating that the potency of Ang2 to support Tie2 activation is lower than Ang1 [25]. Interestingly, at a higher concentration (10 nM), Ang1- and Ang2-mediated PAF synthesis was almost completely lost. This can be explained by the fact that the binding of a ligand to a receptor tyrosine kinase (RTK) induces receptor homo- or heterodimerization, which is essential for the autophosphorylation of tyrosine residues and the initiation of downstream signalling events [26]. However, an overabundance of ligand impedes receptor dimerization [27]. Considering that numerous studies reported the use of angiopoietins, in some cases, at concentrations exceeding 10 nM, our study demonstrates the importance of performing a dose-response curve to establish the suitable concentration to achieve selective biological activities thereby avoiding potentially false interpretations with respect to the biological activities of the angiopoietins.

Recent reports indicate that Tie2 dimerization may be induced to distinctly different extents by Ang1 or Ang2 [28,29], thus it is possible that Ang1 and Ang2, upon binding to Tie2, induce conformational changes in Tie2 resulting in different activation patterns, namely the phosphorylation of different tyrosine residues or activation of different signalling pathways, explaining the differential response between Ang1 and Ang2. Furthermore, we demonstrate that the activation of Tie2 by Ang1 activates p38 and p42/44 MAPKs in a rapid and transient manner and PI3K/Akt for a prolonged period of time. Our data are in line with a previous report demonstrating that upon binding to Tie2, Ang1 activates both p38 and p42/44 MAPKs [18] as well as Akt [30]. Most studies investigating signalling downstream of Tie2 mainly focused on PI3K/Akt [19,31–33] due to the ability of Ang1 to stabilize the vasculature. The ability of Ang1 to activate both proapoptotic (p38 MAPK) and antiapopototic (p42/44 MAPK and PI3K) pathways is not unique since endothelial cell-specific mitogens, such as VEGF, are also capable of activating multiple pathways including p38 and p42/44 MAPKs, and PI3K [34]. Interestingly, we also observed the capacity of Ang2 to activate

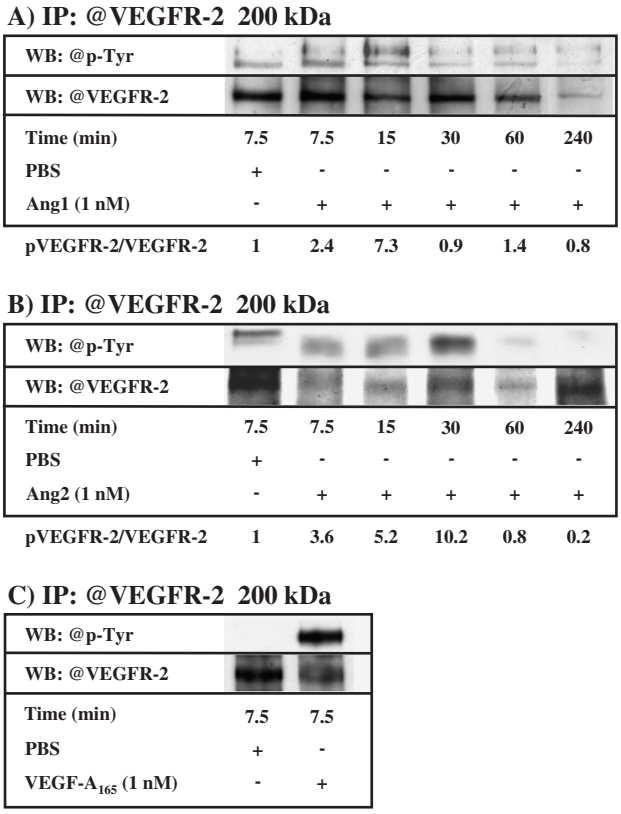

Fig. 7. Treatment of BAEC with Ang1 or Ang2 promotes VEGFR-2 phosphorylation. Confluent BAEC were stimulated with Ang1 or Ang2 (1 nM) for 7.5 min to 4 h. Cell lysates (1 mg) were prepared and immunoprecipitated (IP) with rabbit polyclonal anti-mouse VEGFR-2 IgG. Following resolution by SDS–PAGE and transfer, PVDF membranes were probed with mouse monoclonal antiphosphotyrosine IgG and immunoreactive bands were visualized by chemiluminescence. Membranes were subsequently stripped and VEGFR-2 protein expression was determined following incubation with rabbit polyclonal anti-mouse VEGFR-2 IgG.

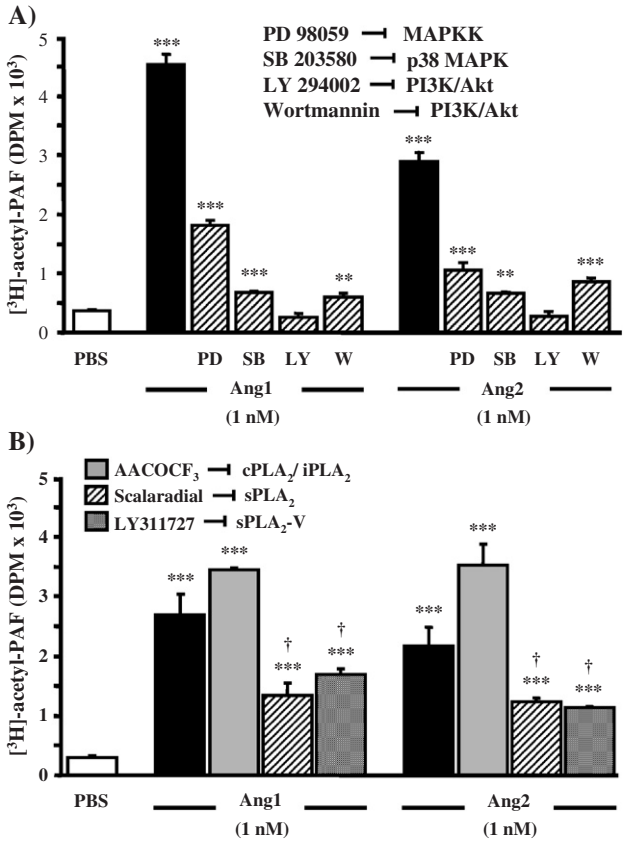

Fig. 8. Angiopoietin-mediated endothelial PAF synthesis requires the activation of p38 MAPK, p42/44 MAPK, and PI3K/Akt signalling pathways and $sPLA_2$-V. (A) Confluent BAEC were pretreated with either PD98059 (10 μM), SB203580 (10 μM), LY294002 (5 μM), or Wortmannin (500 nM) 15 min prior to 4 h of stimulation with Ang1 or Ang2 (1 nM). (B) Confluent BAEC were pretreated with selective PLA_2 pharmacological inhibitors $AACOCF_3$ (10 μM), scalaradial (10 μM), or LY311727 (100 μM) 15 min prior to 4 h of stimulation with Ang1 or Ang2 (1 nM). Values are means±SEM of 3 experiments. $^{**}p<0.01$ and $^{***}p<0.001$ vs. PBS; †$p<0.05$ vs. agonist.

p38 and p42/44 MAPKs, which had yet to be documented, in addition to PI3K/Akt which had previously been described [24,25]. Our observations demonstrate the complex dual nature of Ang2 by its ability to similarly activate p38 and p42/44 MAPK as Ang1. However, Ang2 did not activate Akt in the sustained manner observed with Ang1. This difference may explain in part the ability of Ang2 to destabilize vessels due to an inability to sufficiently activate PI3K and in turn, Akt and focal adhesion kinase (FAK), two crucial elements in the signalling pathway leading to cell survival and migration [35].

4.2. Ang1 and Ang2 induce endothelial PAF synthesis

Our data demonstrate that the angiopoietins constitute a second class of tyrosine kinase receptor ligands with proangiogenic activities. In the present study, we demonstrate that both Ang1 and Ang2 induce endothelial PAF synthesis, however, the profile of PAF synthesis mediated by the angiopoietins is strikingly different to that seen with VEGF-A_{165}. In contrast to what we have previously reported with respect to VEGF-A_{165}-mediated PAF synthesis [3], both angiopoietins induce a rapid, progressive, and sustained endothelial PAF synthesis (maximal within 4 h) whereas VEGF-A_{165} induces a rapid and transient synthesis of PAF [3]. In activated endothelial cells, acute PAF synthesis is mediated through the remodeling pathway and can occur in a very early (2–5 min), early (10–40 min), or delayed (4–8 h) [36] manner. The kinetics observed in the current study follow a biphasic response during which angiopoietins induce an early response which is not as robust as that seen with VEGF-A_{165}. This initial synthesis is followed by a "burst" phase where maximal PAF synthesis is twice as high as the peak observed with VEGF-A_{165}. Based on the kinetics observed, angiopoietin-mediated endothelial PAF synthesis may be complementary to VEGF-mediated PAF synthesis. Perhaps, under inflammatory conditions, VEGF-mediated PAF synthesis provides an initial rapid and transient synthesis followed by the prolonged angiopoietin-mediated response sustaining neutrophil and EC activation leading to endothelial P-selectin translocation and neutrophil adhesion onto EC.

The maximal PAF synthesis observed at 4 h is dependent upon the activation of the p38 MAPK, p42/44 MAPK, and PI3K/Akt signalling pathways. Indeed, pretreatment of BAEC with pharmacological inhibitors for each of the aforementioned pathways resulted in similar inhibition patterns of PAF synthesis mediated by both Ang1 and Ang2. We have recently suggested that the ability of the MAPKK inhibitor to completely block VEGF-A_{165}-mediated endothelial PAF synthesis resides in its ability to prevent PLA_2 activation [5]. Based on our data, it appears that this inhibitor elicits a similar response with respect to angiopoietin-mediated PAF synthesis in BAEC since pretreatment with this inhibitor substantially reduced PAF synthesis. Since p38 MAPK has been shown to directly activate lyso-PAF AT [37], an enzyme essential for PAF synthesis, it is not surprising to observe that p38 MAPK inhibition almost completely abrogated angiopoietin-mediated PAF synthesis. The observation that the PI3K/Akt pathway regulates angiopoietin-mediated PAF synthesis in a positive manner is in stark contrast to what we have previously reported with respect to VEGF-A_{165}-mediated PAF synthesis [5]. Future studies will be required to delineate how the activation of PI3K/Akt pathway modulates downstream effectors involved in both VEGF- and angiopoietin-mediated PAF synthesis.

The phospholipase A_2 family has been implicated in a number of cellular responses and several isoforms of cytosolic ($cPLA_2$), calcium-independent ($iPLA_2$) and secreted ($sPLA_2$) have been identified ([38] for review). As mentioned above, the remodeling pathway of EC PAF synthesis requires the contribution of a PLA_2 to convert membrane phospholipids into lyso-PAF. Having demonstrated that the angiopoietins activate three intracellular signalling pathways known to participate in EC PAF synthesis, the next step was to determine which PLA_2 was implicated in angiopoietin-mediated PAF synthesis. Cytosolic PLA_2 is expressed in most cell types and p42/44 and p38 MAPKs have been implicated in its activation [39–41]. The $iPLA_2$s are the most recently identified members of the PLA_2 superfamily and share the size, intracellular localization, and catalytic mechanisms with $cPLA_2$ [38]. It is apparent that angiopoietin-mediated PAF synthesis is not dependent on $cPLA_2$ and $iPLA_2$ as pretreatment with a specific $cPLA_2$ and $iPLA_2$ inhibitor, $AACOCF_3$ did not

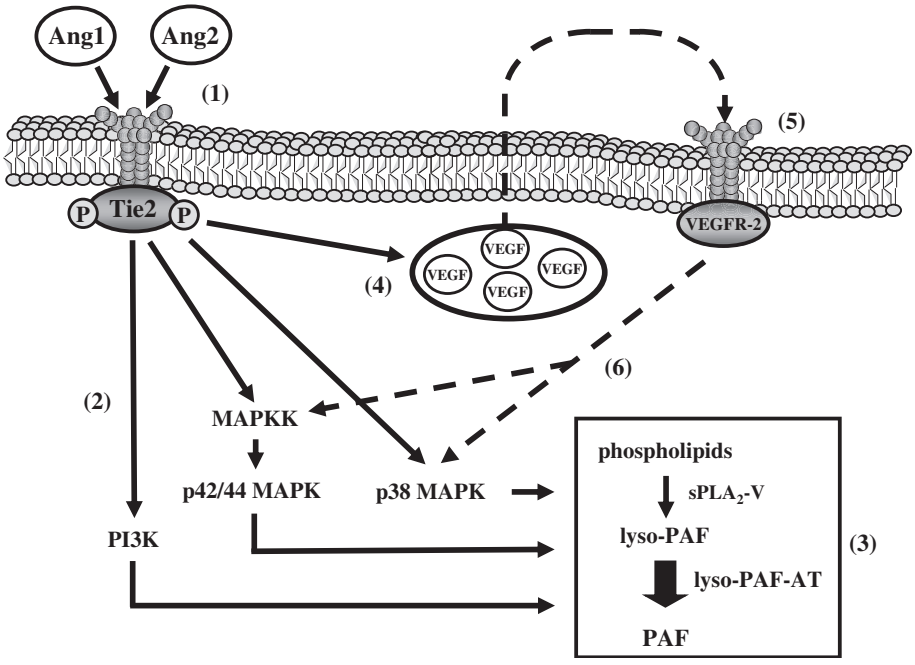

Fig. 9. Proposed mechanism by which angiopoietins mediate endothelial PAF synthesis. Stimulation of tyrosine kinase Tie2 receptor by Ang1 or Ang2 (1) activates PI3K, p42/44 MAPK, and p38 MAPK pathways (2). These intracellular signalling pathways, through the action of $sPLA_2$-V, induce endothelial PAF synthesis (3). Concurrently, Tie2 activation, through a mechanism yet to be defined, promotes the relocalization of endogenous VEGF to the cell membrane (4) where it can bind to one of its receptors, VEGFR-2 (5). Activation of VEGFR-2 leads to p38 and p42/44 MAPKs activation and endothelial PAF synthesis (6) which in turn potentiates angiopoietin-mediated PAF synthesis.

prevent but even slightly increased EC PAF synthesis by both Ang1 and Ang2 at 4 h. We have previously reported that $sPLA_2$-V is implicated in VEGF-A_{165}-mediated EC PAF synthesis [4] and thus opted to target this particular $sPLA_2$ isoform. Using pharmacological inhibitors, we demonstrated that pretreatment of BAEC with a non-specific $sPLA_2$ inhibitor, scalaradial, blocked angiopoietin-mediated PAF synthesis by approximately 50%. In addition, LY311727 at a concentration (100 μM) known to specifically block $sPLA_2$-IIA and -V activity similarly inhibited angiopoietin-mediated PAF synthesis. Since $sPLA_2$-IIA is not expressed in BAEC [4], this suggests the essential contribution of $sPLA_2$-V in angiopoietin-mediated PAF synthesis. Therefore, it is interesting to note that although angiopoietins have a different PAF synthesis profile than VEGF-A_{165}, both require the same phospholipase, thereby bestowing a critical role upon $sPLA_2$-V in EC PAF synthesis.

The peak in angiopoietin-mediated PAF synthesis, could be representative of a "delayed" PAF production as described previously ([36] for review) and hence require newly synthesized proteins for cell activation. Since BAEC stimulation with VEGF-A_{165} induces PAF synthesis and that EC express VEGF [20–23], we sought to investigate whether VEGF was implicated in angiopoietin-mediated PAF synthesis. First, we did not detect an upregulation of VEGF mRNA by RT-PCR analysis when BAEC were stimulated with Ang1 or Ang2 (data not shown) nor did we see significant fluctuations in the quantity of endogenous VEGF by ELISA. We also observed that no or marginal amounts of VEGF were released into the supernatant. However, when BAEC were pretreated with VEGF receptor inhibitors prior to stimulation with Ang1 or Ang2, angiopoietin-mediated EC PAF synthesis was inhibited by approximately 50%. This partial reduction of PAF synthesis was not due to a non-specific inhibition of Tie2 activation by VTK and SU1498 since we observed by Western blot analysis that these inhibitors of VEGF receptors did not alter Tie2 phosphorylation mediated by angiopoietins but prevented VEGF-A_{165}-mediated VEGFR-1 and R-2 activation [7,16] and PAF synthesis. We then postulated that endogenous VEGF was being shuffled from the intracellular compartment to the endothelial cell surface membrane to interact with its cell surface membrane receptors and contribute to angiopoietin-induced PAF synthesis. This hypothesis was confirmed by confocal microscopy whereby we observed the presence of a significant amount of endogenous VEGF at the cell surface within 7.5 min of stimulation with Ang1, and to a lesser extent with Ang2, which remained noticeable up to 4 h post-stimulation with both angiopoietins. Furthermore, we observed that a treatment with Ang1 or Ang2 was capable of mediating VEGFR-2 phosphorylation. The reduced capacity of Ang2 to promote PAF synthesis may be related to its less intense activation of Tie2 and VEGF relocalization on endothelial cell surface membrane. Our data are in line with previous studies reporting that a treatment of bovine aortic endothelial cells, namely with sphingosine 1-phosphate (S1P), can lead to VEGFR-2 phosphorylation and activation of downstream effectors [42]. To the best of our knowledge, our study is the first one to demonstrate the capacity of angiopoietins to induce VEGFR-2 phosphorylation and biological activities such as PAF synthesis.

Based on our current observations as well as previous studies, it appears that under specific conditions, both Ang1 and Ang2 are

capable of mediating proinflammatory events. We have recently reported that the angiopoietins are capable of promoting endothelial P-selectin translocation and the adhesion of neutrophils onto activated human umbilical vein endothelial cells (HUVEC) as well as activating Tie2 receptors on neutrophils leading to PAF synthesis promoting a rapid upregulation of the β_2-integrin complex (CD11/CD18) and contributing to an increase in neutrophil adhesion onto activated EC thereby demonstrating that the angiopoietins should be considered as acute proinflammatory mediators [13]. However, in the aforementioned study, angiopoietins did not induce PAF synthesis in HUVEC [13] whereas we demonstrate their powerful capacity to mediate PAF synthesis in BAEC, thereby suggesting tissue specificity. We have previously observed that BAEC induce a more robust endothelial PAF synthesis than HUVEC [4]. Recent studies reported that Ang1 possesses anti-inflammatory properties. For instance, under in vivo conditions, Ang1 has been shown to prevent VEGF-mediated vascular permeability [10,43,44] and in vitro it reduces the basal activation of vascular endothelial cadherin (VE-cadherin) and β-catenin, concomitantly with a reduction of VEGF-mediated endothelial cell permeability [45,46]. Interestingly, the above studies utilized HUVEC and it is therefore possible that the anti-inflammatory effects attributed to Ang1 under in vitro conditions stem from the inability of angiopoietins to promote PAF synthesis in this endothelial subtype.

In summary, our study demonstrates for the first time that Ang1 and Ang2 are both capable of inducing endothelial PAF synthesis and this in a temporal resolution different than the rapid and transient PAF synthesis induced by VEGF-A_{165}. Furthermore, this synthesis requires the activation of the p38 MAPK, p42/44 MAPK, and PI3K/Akt intracellular signalling pathways as well as the induction of $sPLA_2$-V. In addition, angiopoietin-mediated endothelial PAF synthesis is partly regulated by a redistribution of endogenous VEGF to the cell surface membrane which may subsequently potentiate endothelial PAF synthesis (Fig. 9). In our study, Ang2 behaved as a partial to full Tie2 agonist in function of its concentration, further strengthening the current view of tissue and context specificity with respect to Ang2 activity. Taken together, our results demonstrate that the angiopoietins, like VEGF, constitute another family of angiogenic growth factors capable of promoting proinflammatory events.

Acknowledgments

Dr. Sirois is a Scholar from the Canadian Institutes of Health Research (CIHR), and this work was supported by grants from the CIHR (MOP-43919) and Québec Heart and Stroke Foundation (M.G.S.). We would like to thank Mr. Louis R. Villeneuve for his technical assistance and Dr. Jerome Fleisch for the generous donation of LY311727.

References

[1] P. Carmeliet, Nat. Med. 6 (4) (2000) 389.
[2] H.F. Dvorak, L.F. Brown, M. Detmar, A.M. Dvorak, Am. J.Pathol. 146 (5) (1995) 1029.
[3] M.G. Sirois, E.R. Edelman, Am. J. Physiol. 272 (6 Pt 2) (1997) H2746.
[4] P.N. Bernatchez, M.V. Winstead, E.A. Dennis, M.G. Sirois, Br. J. Pharmacol. 134 (1) (2001) 197.
[5] P.N. Bernatchez, B.G. Allen, D.S. Gelinas, G. Guillemette, M.G. Sirois, Br. J. Pharmacol. 134 (6) (2001) 1253.
[6] G. Montrucchio, E. Lupia, E. Battaglia, L. Del Sorbo, M. Boccellino, L. Biancone, G. Emanuelli, G. Camussi, Arterioscler. Thromb. Vasc. Biol. 20 (1) (2000) 80.
[7] S. Rollin, C. Lemieux, R. Maliba, J. Favier, L.R. Villeneuve, B.G. Allen, S. Soker, N.G. Bazan, Y. Merhi, M.G. Sirois, Blood 103 (10) (2004) 3789.
[8] S. Davis, T.H. Aldrich, P.F. Jones, A. Acheson, D.L. Compton, V. Jain, T.E. Ryan, J. Bruno, C. Radziejewski, P.C. Maisonpierre, G.D. Yancopoulos, Cell 87 (7) (1996) 1161.
[9] P.C. Maisonpierre, C. Suri, P.F. Jones, S. Bartunkova, S.J. Wiegand, C. Radziejewski, D. Compton, J. McClain, T.H. Aldrich, N. Papadopoulos, T.J. Daly, S. Davis, T.N. Sato, G.D. Yancopoulos, Science 277 (5322) (1997) 55.
[10] G. Thurston, C. Suri, K. Smith, J. McClain, T.N. Sato, G.D. Yancopoulos, D.M. McDonald, Science 286 (5449) (1999) 2511.
[11] K. Teichert-Kuliszewska, P.C. Maisonpierre, N. Jones, A.I. Campbell, Z. Master, M.P. Bendeck, K. Alitalo, D.J. Dumont, G.D. Yancopoulos, D.J. Stewart, Cardiovasc. Res. 49 (3) (2001) 659.
[12] Y. Mochizuki, T. Nakamura, H. Kanetake, S. Kanda, J. Cell Sci. 115 (Pt 1) (2002) 175.
[13] C. Lemieux, R. Maliba, J. Favier, J.F. Theoret, Y. Merhi, M.G. Sirois, Blood 105 (4) (2005) 1523.
[14] P.N. Bernatchez, S. Soker, M.G. Sirois, J. Biol. Chem. 274 (43) (1999) 31047.
[15] L.F. Hennequin, A.P. Thomas, C. Johnstone, E.S. Stokes, P.A. Pl inverted question marke, J.J. Lohmann, D.J. Ogilvie, M. Dukes, S.R. Wedge, J.O. Curwen, J. Kendrew, C. Lambert-van der Brempt, J. Med. Chem. 42 (26) (1999) 5369.
[16] P.E. Neagoe, C. Lemieux, M.G. Sirois, J. Biol. Chem. 280 (11) (2005) 9904.
[17] J.L. Arbiser, H. Larsson, L. Claesson-Welsh, X. Bai, K. LaMontagne, S.W. Weiss, S. Soker, E. Flynn, L.F. Brown, Am. J. Pathol. 156 (4) (2000) 1469.
[18] R. Harfouche, J.P. Gratton, G.D. Yancopoulos, M. Noseda, A. Karsan, S.N. Hussain, FASEB J. 17 (11) (2003) 1523.
[19] I. Kim, H.G. Kim, J.N. So, J.H. Kim, H.J. Kwak, G.Y. Koh, Circ. Res. 86 (1) (2000) 24.
[20] M. Inoue, H. Itoh, T. Tanaka, T.H. Chun, K. Doi, Y. Fukunaga, N. Sawada, J. Yamshita, K. Masatsugu, T. Saito, S. Sakaguchi, M. Sone, K. Yamahara, T. Yurugi, K. Nakao, Arterioscler. Thromb. Vasc. Biol. 21 (4) (2001) 560.
[21] R. Pai, B. Soreghan, I.L. Szabo, M. Pavelka, D. Baatar, A.S. Tarnawski, Nat. Med. 8 (3) (2002) 289.
[22] D. Wang, H.J. Huang, A. Kazlauskas, W.K. Cavenee, Cancer Res. 59 (7) (1999) 1464.
[23] S.G. Robbins, J.R. Conaway, B.L. Ford, K.A. Roberto, J.S. Penn, Growth Factors 14 (4) (1997) 229.
[24] I. Kim, J.H. Kim, S.O. Moon, H.J. Kwak, N.G. Kim, G.Y. Koh, Oncogene 19 (39) (2000) 4549.
[25] S. Kanda, Y. Miyata, Y. Mochizuki, T. Matsuyama, H. Kanetake, Cancer Res. 65 (15) (2005) 6820.
[26] P. van der Geer, T. Hunter, R.A. Lindberg, Annu. Rev. Cell. Biol. 10 (1994) 251.
[27] B.L. Gruber, M.J. Marchese, R. Kew, Blood 86 (7) (1995) 2488.
[28] C.H. Heldin, Cell 80 (2) (1995) 213.
[29] J. Schlessinger, Cell 103 (2) (2000) 211.
[30] C.D. Kontos, T.P. Stauffer, W.P. Yang, J.D. York, L. Huang, M.A. Blanar, T. Meyer, K.G. Peters, Mol. Cell. Biol. 18 (7) (1998) 4131.
[31] N. Jones, Z. Master, J. Jones, D. Bouchard, Y. Gunji, H. Sasaki, R. Daly, K. Alitalo, D.J. Dumont, J. Biol. Chem. 274 (43) (1999) 30896.
[32] L.M. DeBusk, D.E. Hallahan, P.C. Lin, Exp. Cell Res. 298 (1) (2004) 167.
[33] I. Kim, H.G. Kim, S.O. Moon, S.W. Chae, J.N. So, K.N. Koh, B.C. Ahn, G.Y. Koh, Circ. Res. 86 (9) (2000) 952.
[34] Y. Yu, J.D. Sato, J. Cell. Physiol. 178 (2) (1999) 235.

[35] G.Y. Koh, I. Kim, H.J. Kwak, M.J. Yun, J.C. Leem, Exp. Mol. Med. 34 (1) (2002) 1.
[36] F. Bussolino, G. Camussi, Eur. J. Biochem. 229 (2) (1995) 327.
[37] A.B. Nixon, J.T. O'Flaherty, J.K. Salyer, R.L. Wykle, J. Biol. Chem. 274 (9) (1999) 5469.
[38] S. Chakraborti, Cell. Signal 15 (7) (2003) 637.
[39] L.L. Lin, M. Wartmann, A.Y. Lin, J.L. Knopf, A. Seth, R.J. Davis, Cell 72 (2) (1993) 269.
[40] R.A. Nemenoff, S. Winitz, N.X. Qian, V. Van Putten, G.L. Johnson, L.E. Heasley, J. Biol. Chem. 268 (3) (1993) 1960.
[41] R.M. Kramer, E.F. Roberts, S.L. Um, A.G. Borsch-Haubold, S.P. Watson, M.J. Fisher, J.A. Jakubowski, J. Biol. Chem. 271 (44) (1996) 27723.
[42] T. Tanimoto, Z.G. Jin, B.C. Berk, J. Biol. Chem. 277 (45) (2002) 42997.
[43] G. Thurston, J.S. Rudge, E. Ioffe, H. Zhou, L. Ross, S.D. Croll, N. Glazer, J. Holash, D.M. McDonald, G.D. Yancopoulos, Nat. Med. 6 (4) (2000) 460.
[44] Y. Wang, S. Pampou, K. Fujikawa, L. Varticovski, J. Cell. Physiol. 198 (1) (2004) 53.
[45] J.R. Gamble, J. Drew, L. Trezise, A. Underwood, M. Parsons, L. Kasminkas, J. Rudge, G. Yancopoulos, M.A. Vadas, Circ. Res. 87 (7) (2000) 603.
[46] L. Pizurki, Z. Zhou, K. Glynos, C. Roussos, A. Papapetropoulos, Br. J. Pharmacol. 139 (2) (2003) 329.