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Regulation of the proinflammatory properties of angiopoietins

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Regulation of the proinflammatory properties of angiopoietins

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Résumé

La découverte récente des angiopoïétines (Ang1 et Ang2) a permis de démontrer qu'elles peuvent, via leur liaison au récepteur Tie2, moduler la perméabilité et la stabilité vasculaire, et ainsi contribuer à diverses étapes du processus angiogénique. Nous avons démontré que les angiopoïétines sont capables de moduler des processus proinflammatoires dans les cellules endothéliales (CE), notamment la synthèse du facteur d'activation plaquettaire (PAF) et la translocation de la P-sélectine.

Le *vascular endothelial growth factor* (VEGF) est le seul facteur angiogénique capable d'induire une réponse inflammatoire et cet effet inflammatoire est médié par la synthèse endothéliale du PAF. De plus, la synthèse de PAF endothéliale induite par le VEGF-A_{165} implique l'activation des voies de signalisation des p38 et p42/44 *mitogen-activated protein kinases* (MAPK). Sachant que l'inflammation joue un rôle important dans l'angiogénèse et que les angiopoïétines agissent de concert avec le VEGF dans la modulation de la plasticité vasculaire, nous avons donc étudié la capacité des angiopoïétines d'induire la synthèse du PAF et les mécanismes intracellulaires par lesquels cette synthèse se produit dans les CE. Nous avons observé qu'Ang1 et Ang2 induisent la phosphorylation rapide et transitoire de Tie2, des voies p42/44 et p38 MAPK ainsi que PI3K/Akt et finalement la synthèse de PAF de façon dose- et temps-dépendante dans les cellules d'aortes bovines (BAEC). L'effet maximal d'Ang1 et Ang2 sur la synthèse de PAF est obtenu après 240 minutes de stimulation (1695% et 851% d'augmentation respectivement). La synthèse de PAF médiée par les angiopoïétines

nécessite l'activation des voies de signalisation intracellulaires des p38 MAPK, p42/44 MAPK et PI3K ainsi que d'une phospholipase A_2 sécrétée de type V ($sPLA_2$-V). De plus, nos résultats démontrent que la synthèse de PAF endothéliale induite par les angiopoïétines est en partie médiée par une relocalisation de VEGF endogène vers la membrane cellulaire.

Nous avons récemment démontré que le PAF est impliqué dans la translocation de la P-sélectine et l'adhésion de neutrophiles sur les CE induite par le VEGF-A_{165} et que Ang1 et Ang2 étaient capables de promouvoir la translocation de la P-sélectine endothéliale et l'adhésion de neutrophiles sur des CE. Par conséquent, nous avons voulu déterminer les mécanismes impliqués et le rôle du PAF endogène dans la translocation de la P-sélectine endothéliale par les angiopoïétines. Nous avons observé que Ang1 et Ang2 (10^{-9} M) pouvaient induire une activation rapide du récepteur Tie2 et une translocation rapide et transitoire (maximale à 7,5 minutes et correspondant à des augmentations de 125% et 100% sur les valeurs témoins, respectivement) de la P-sélectine. De plus, nous avons décrit pour la première fois que la translocation de la P-sélectine endothéliale induite par les angiopoïétines dépend du calcium en plus d'être régulée par l'activation de la phospholipase C-γ (PLC-γ).

En conclusion, nos résultats démontrent que les angiopoïétines possèdent la capacité de moduler des effets proinflammatoires tels la synthèse de PAF et la translocation de la P-sélectine au niveau de l'endothélium.

Mots clés: Angiopoïétines, inflammation, angiogénèse, facteur d'activation plaquettaire (PAF), P-sélectine, Tie2.

Summary

Recently discovered, Ang1 and Ang2, upon binding to the Tie2 tyrosine kinase receptor, modulate vascular permeability and integrity, contributing to angiogenesis. Herein, we demonstrated that angiopoietins are capable of modulating proinflammatory processes in endothelial cells (EC), namely the synthesis of platelet-activating factor (PAF) and P-selectin translocation. In addition, we also identified the intracellular mechanisms responsible for these biological effects.

Vascular endothelial growth factor (VEGF) is the only angiogenic growth factor capable of inducing an inflammatory response and we have demonstrated that this effect is mediated by the endothelial synthesis of PAF. Furthermore, VEGF-A_{165}-mediated endothelial PAF synthesis requires the activation of both p38 and p42/44 mitogen-activated protein kinases (MAPK). Since inflammation exists in a mutually-dependent association with angiogenesis and angiopoietins act in concert with VEGF to modulate vascular plasticity during postnatal neovascularization, we sought to determine whether Ang1 and/or Ang2 were capable of inducing endothelial PAF synthesis and if so, what mechanisms were implicated. Treatment of bovine aortic endothelial cells (BAEC) with Ang1 or Ang2 (10^{-9} M) induced a rapid Tie2 phosphorylation and a rapid, progressive and sustained endothelial PAF synthesis maximal within 240 minutes (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.

We have recently reported that VEGF-A_{165} inflammatory effects are mediated through the synthesis of PAF by endothelial cells which contributes to the induction of endothelial P-selectin translocation and neutrophil adhesion onto activated EC. Furthermore, our laboratory demonstrated that Ang1 and Ang2 are also both capable of promoting endothelial P-selectin translocation and neutrophil adhesion onto EC. We therefore sought to investigate the cellular mechanisms implicated in angiopoietin-mediated P-selectin translocation in BAEC and assess the role of PAF in this process. We observed that Ang1 and Ang2 (10^{-9} M) are both capable of mediating a rapid Tie2 phosphorylation as well as a rapid and transient endothelial P-selectin translocation maximal within 7.5 minutes (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 (PLC-γ) activation.

In conclusion, our data demonstrate that angiopoietins are capable of modulating proinflammatory events, namely endothelial PAF synthesis and P-selectin translocation in BAEC and shed light on the intracellular pathways through which angiopoietins regulate these events.

Keywords: Angiopoietins, inflammation, angiogenesis, platelet-activating factor (PAF), P-selectin, Tie2.

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Article #2

List of Abbreviations

AA: arachidonic acid

Ang1, Ang2: angiopoietin-1 and -2

BAEC: bovine aortic endothelial cells

$cPLA_2$: cytosolic phospholipase A_2

DAG: diacylglycerol

EC: endothelial cells

eNOS: endothelial nitric oxide synthase

ESL-1: E-selectin ligand-1

FGF: fibroblast growth factor

HIF: hypoxia-inducible factor

HUVEC: human umbilical vein endothelial cells

ICAM-1: intercellular adhesion molecule-1

IL: interleukin

IP_3: inositol 1,4,5-triphosphate

$iPLA_2$: calcium-independent phospholipase A_2

LPS: lipopolysaccharide

Lyso-PAF AT: lyso-PAF acetyl transferase

MAPK: mitogen-activated protein kinase

NO: nitric oxide

NRP-1: neuropilin-1

PAF: platelet-activating factor

PAFR: platelet-activating factor receptor

PECAM-1: platelet-endothelial cellular adhesion molecule-1

PGI_2: prostaglandin I_2 (prostacyclin)

PI3K: phosphatidyl inositol-3 kinase

PLC-γ: phospholipase C-γ

PKC: protein kinase C

PlGF: placental growth factor

PMA: phorbol myristate acetate

PMN: polymorphonuclear cells

PSGL-1: P-selectin glycosylated ligand-1

RTK: receptor tyrosine kinase

$sPLA_2$: secreted phospholipase A_2

Tie1, Tie2: tyrosine kinase with immunoglobulin and epidermal growth factor homology domains receptor-1 and -2

VCAM-1: vascular cell adhesion molecule-1

VE-cadherin: vascular-endothelial cadherin

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

WPB: Weibel-Palade bodies

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1.0 Introduction

1.1 Biology of the vascular endothelium

1.1.1 Role of the endothelium in vascular homeostasis

The vascular system has fascinated mankind for thousands of years. Egyptian physicians recognized that 'there were vessels in him for every part of the body' [1], Aristotle considered that 'blood vessels are like watercourses in gardens: they start from one spring and branch off into numerous channels so as to supply every part of the garden' [1], and Leonardo da Vinci (1452-1519) speculated that the vasculature developed like a tree from a seed (the heart) by sprouting roots (capillary network) and a trunk with major branches (the aorta and arteries) [2]. Indeed, the complexity of the vascular system prompted the formulation of several hypotheses in an attempt to understand its functioning and regulation. However, the defence of these postulates was rendered difficult by the absence of anatomic proof. William Harvey (1578-1657) was the first physiologist to describe the circulation of blood in large vessels [3] yet he could not explain how blood passed from the arteries to the veins. Thirty years later, Malpighi (1628-1694) observed, through the newly developed microscope, the interconnecting capillary network. Pioneering work conducted by Ruysch, (1638-1731) enabled the development of techniques permitting the identification of the micro- and macroscopic constituents of blood vessels enabling anatomic research to progress.

The cellular monolayer carpeting the luminal aspect of blood vessels attracted the attention of various researchers. The term endothelium was coined by His in 1865 who believed that this layer served solely as a physical barrier to prevent blood from flowing into the surrounding tissues [4]. However, others believed that the endothelium played a crucial role in homeostasis, namely preventing the formation of thrombi and the invasion of smooth muscle cells (SMC) into the vascular lumen [4,5]. Due to their direct contact with blood, endothelial cells perform a critical role in all aspects of tissue homeostasis. Endothelial cells regulate vascular tone through interaction with components of the peripheral nervous system and are involved in thrombolysis and blood coagulation. Endothelial cells are also implicated in inflammatory and immunological processes. The endothelial cells surface consists of a layer of surface glycoprotein (glycocalyx) that provides not only a local charged barrier to the transendothelial migration of blood cells and plasma proteins under normal physiological conditions but is also physiologically active [6]. Cells within the vessel wall communicate with surrounding cells, can sense changes in pressure and flow, and can secrete molecules that perform various functions. The belief that endothelial cells (EC) were merely barriers between blood and the interstitium was revolved.

1.1.2 Role of the endothelium in the pathogenesis of vascular disorders

Despite their role in the maintenance of vascular homeostasis, endothelial cells may also play a deleterious role under certain situations such as inflammation. British

surgeon John Hunter (1728-1793) reported that the characteristic redness associated with inflammation is due to increased blood flow through dilated vessels. Early work placed an emphasis on the fact that inflammation is characterized by an endothelium-dependent alteration in the vasculature. Furthermore, the endothelium was directly associated with the accumulation and transmigration of leukocytes into underlying tissues [5,7].

Recent studies have demonstrated the crucial role played by the endothelium in the development of various types of cancer. In fact, early studies reported that cancerous tumours contain a high density network of blood vessels that supply the rapidly growing cancerous cells with the necessary oxygen and nutrients and that the disruption or inhibition of this vascular network may be a key in the treatment of cancer [8]. It is now commonly accepted that the unorganized growth of new blood vessels is present in other pathologies such as atherosclerosis, retinopathies, psoriasis, and rheumatoid arthritis [9-11]. Thus, the endothelium possesses a dual nature. On one hand, the endothelium plays a predominant role in the maintenance of homeostasis yet it can regulate the progression of various pathologies.

1.2 Angiogenesis

1.2.1 Overview

Angiogenesis is defined as the formation of blood vessels from pre-existing ones. Tissue and cells require blood to supply the nutrients and oxygen required for homeostasis and growth. In 1935, Arthur Tremain Hertig (1904-1990) described

angiogenesis in the placenta of pregnant monkeys. Classic angiogenesis, or sprouting angiogenesis, has been extensively studied and its stepwise progression was initially detailed by Dr. Folkman in 1971 [10]. Angiogenesis initiates with vasodilation and an increase in vascular permeability in response to local hypoxia. Hypoxia is an important stimulus for expansion of the vascular bed once tissues are no longer oxygenated by simple diffusion. Hypoxia triggers angiogenesis through the hypoxia-indicible factor (HIF-1α). HIF is an αβ heterodimer initially identified as a DNA binding factor implicated in erythropoietin hypoxia-inducible activity [12]. HIF-1α subunits are inducible by hypoxia and are able to interact with hypoxia-responsive element to induce transcriptional activity. Under normoxia, HIF-1α is rapidly inactivated by proteolysis but in a hypoxic environment, HIF-1α mediates the increase in expression of various angiogenic factors such as vascular endothelial growth factor (VEGF) which increases vascular permeability. The ensuing protein extravasation of plasma proteins lays down a provisional scaffold for migrating endothelial cells [13]. The increase in permeability is mediated by the formation of fenestrations and a redistribution of platelet endothelial cell adhesion molecule (PECAM) and vascular endothelial (VE)-cadherin. The intercellular junctions are loosened and the endothelial cells may disengage from supporting structures (pericytes or smooth muscle cells) in response to the degradation of the basement membrane and the extracellular matrix. Pericytes, or perivascular cells surround and provide endothelial cells, which lack their own blood supply, with a balanced cellular microenvironment. Liberated, the proliferating cells can then migrate to the site of neovascularization and form tubular structures that mature through vessel

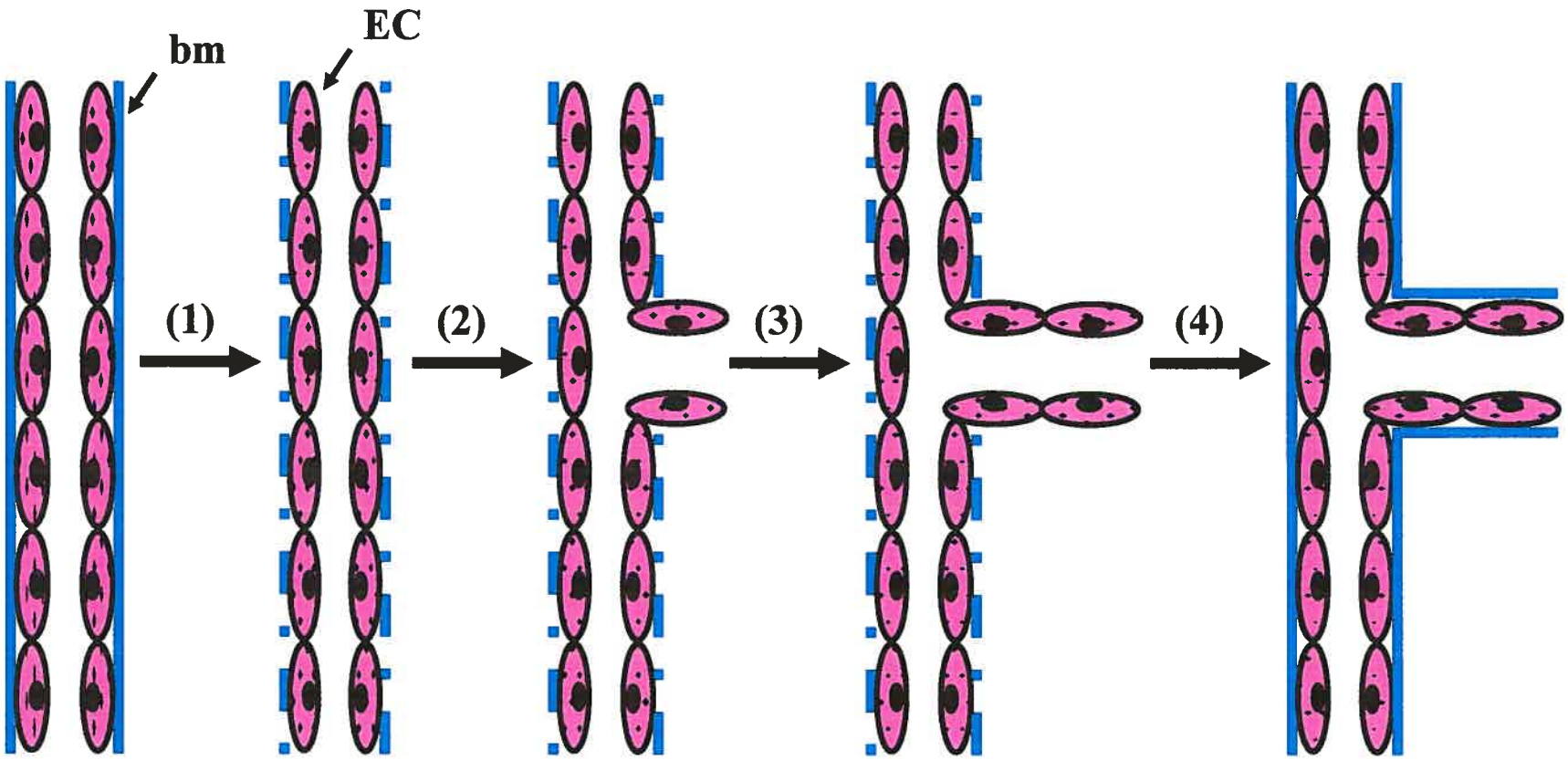

Figure 1: The stepwise progression of budding (classical) angiogenesis. The destabilization of the basement membrane (bm) (1) allows for the migration (2) and proliferation of endothelial cells (EC) (3) which can then differentiate and form tubular structures (4). (adapted from Savona *et al.* Rev Prat. 1997; 47: 2239-43)

remodelling. Once assembled into vessels, endothelial cells become quiescent and survive for years. A summary of classic angiogenesis appears in Figure 1.

1.2.2 Physiological angiogenesis

In the embryo, endothelial progenitors originating from the differentiation of angioblasts, assemble in a primitive capillary plexus in a process termed vasculogenesis. After the primary capillary plexus is formed, more endothelial cells are generated and may form new capillaries through angiogenesis. Endothelial cells can differentiate into either arterial or venous cells in embryonic development indicating remarkable phenotypic plasticity [14]. Angiogenesis is a necessary physiological process in embryonic development whereas neovascularization in the adult occurs under specific conditions.

Angiogenesis is commonly observed during wound healing where sprouting induces the formation of new capillaries from pre-existing ones. In adults, endothelial cells differentiate from endothelial progenitor cells (EPC), mesoangioblasts, multipotent adult progenitor cells, or side-population cells in bone marrow [15]. During the formation of new connective tissue, hypervascularization is observed and once the injury repaired, most of the neovessels regress and normal vascularization is restored. During prolonged physical efforts, localized hypoxia may occur in skeletal muscle in response to the increased oxygen demand and this situation may be compensated for, in the long-term, through the formation of new vessels [16]. Lastly, the female reproductive system consists of the only tissues whose vasculature undergoes regular

growth and regression. Upon ovulation and follicular rupture, the remaining cells form the *corpus luteum* and secrete estradiol and progesterone. The ensuing growth and maturation of the *corpus luteum* results in an intense vascular development and at the end of the menstrual cycle, this vascular network regresses. In unison, the uterine lining also undergoes angiogenesis in preparation for the potential implantation of the embryo. In absence of fertilization, the endometrial vasculature regresses.

1.2.3 Pathological angiogenesis

Dysregulated vessel growth may have a significant impact on health and contribute to the pathogenesis of many disorders. Indeed, numerous disorders are characterized or caused by excessive angiogenesis including cancer, psoriasis, rheumatoid arthritis, retinopathies, and atherosclerosis ([1] for review). Interestingly, a high fat diet has been demonstrated to promote angiogenesis in adipose tissue and angiogenic factors stimulate adipogenesis [17,18]. Other disorders may be attributable to abnormal vessel regression and maturation such as purpura, preeclampsia, nephropathy, and impaired reendothelialization after arterial injury [1]. If blood supply is impaired, tissue ischemia may result leading to patient death or disability. Conversely, excessive vessel growth could lead to increased tumour growth or inflammation. An in-depth understanding of the molecular mechanisms of angiogenesis is of paramount importance for the development of therapeutic strategies to combat inflammatory, malignant, and ischaemic disorders. Natural inhibitors of angiogenesis, such as angiostatin and endostatin [19,20] have been identified and soluble receptors of angiogenic factors have been shown to efficiently block tumour angiogenesis and

growth [21,22]. However, efforts to therapeutically generate new blood vessels have not been as successful as those to inhibit angiogenesis and strategies must be explored.

1.3 Regulation of angiogenesis

The aforementioned angiogenic processes are subject to a tight and rigorous control at the molecular level. The consensus is that the regulated induction, stabilization, and regression of the vasculature are the result of a balance between angiogenic and angiostatic elements. Once the equilibrium is disrupted and the balance shifts, angiogenesis is either induced or repressed. Until recently, vascular endothelial growth factor (VEGF) was the only growth factor proven to be specific to and critical for blood vessel formation [11,23]. Other growth factors, such as fibroblast growth factor (FGF) had effects in endothelial cell assays [2] but also acted on many other cell types. New growth factors, namely the angiopoietins, acting on the vascular endothelium in a tightly regulated complementary and coordinated manner have recently been identified [24,25].

1.3.1 Vascular endothelial growth factor (VEGF-A)

Initially identified as vascular permeability factor (VPF) in the 1980s because tumour growth is associated with increased microvascular permeability, VEGF is the most important modulator of vascular formation. The gene coding for VEGF-A is composed of 8 exons separated by 7 introns (Figure 2). Alternate splicing gives rise

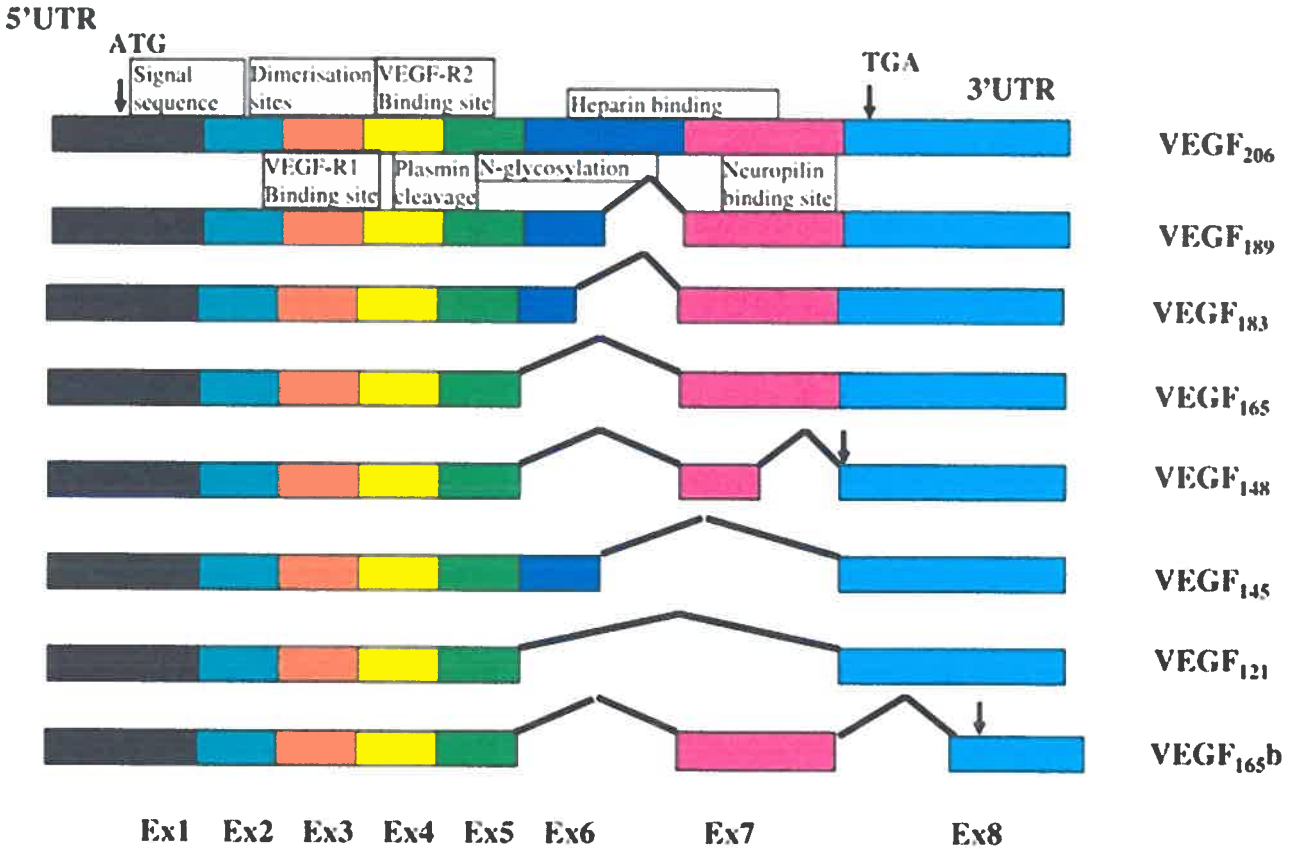

Figure 2: VEGF-A isoforms. The gene coding for VEGF-A produces several isoforms through alternative splicing. The presence of exons 6 and 7 confers upon VEGF-A_{206} complete sequestration within the extracellular matrix. The ability of VEGF-A_{165} to bind to the neuropilin-1 (NRP-1) coreceptor stems from it having exon 7, whereas it is absent in the sequence coding for VEGF-A_{121}. (from Bates *et al.*, Vascular Pharmacology, 2002; 39: 225-37)

to 6 different isoforms of VEGF-A respectively comprising 206, 189, 183, 165, 145, or 121 amino acids [26-28]. These isoforms differ from one another by the presence or absence of sequences encoded by exons 6 and 7, sequences responsible for binding to heparin and the extracellular matrix. VEGF-A_{165}, the most abundant and potent isoform, does not possess exon 6 and displays a moderate affinity for heparin which explains why 50 to 70% of secreted VEGF-A_{165} remains bound to cells or the extracellular matrix [29]. On the other hand, VEGF-A_{121} has neither exon 6 or 7 and does not bind heparin and is thus free to diffuse in the circulation [30]. In addition, the lack of exon 7 also prevents VEGF-A_{121} from binding to the neuropilin-1 (NRP-1) coreceptor and thus it is less powerful than VEGF-A_{165} at inducing biological effects. In contrast, VEGF-A_{189} and $-A_{206}$ possess exons 6 and 7 and thus are completely sequestered with the extracellular matrix [31].

Other members of the VEGF family were identified based on their homology to VEGF-A: placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and VEGF-E [32-35]. Placental growth factor and VEGF-B both play a predominant role in embryonic development [32]. PlGF knockout mice do not have an apparent phenotype. However, these mice recover poorly from experimental myocardial infarction and exhibit impaired collateral formation in response to hind limb ischemia [36]. Overexpression of PlGF in the skin of transgenic mice results in a hypervascular phenotype with increased inflammatory and permeability responses [37,38], while local administration of PlGF with recombinant adenoviruses or as a recombinant protein induces the formation of mature, leakage-resistant vessels in a macrophage-

dependent manner [39]. The precise role of VEGF-B *in vivo* is not known. Since VEGF-B is highly expressed in striated muscle, myocardium and brown fat [40,41], its function may be linked to high cellular energy metabolism.

On the other hand, VEGF-C and –D are synthesized as precursors that may be metabolized into by-products with various properties [42,43]. VEGF-C is expressed predominantly in regions where lymphatic vessels develop [44,45]. The expression and mice lacking both VEGF-C alleles fail to develop lymphatic vessels and succumb to tissue edema at E15.5–E17.5 [44]. VEGF-D is present in most human tissues, most abundantly in the lung and skin during embryogenesis [46]. In experimental tumours VEGF-D increases lymphatic vessel growth and lymphatic metastasis [47]. VEGF-D is expressed by melanoma cells and has been proposed to have a role in tumour angiogenesis and lymphangiogenesis in this tumour [48]. VEGF-E, identified in the genome of the Orf virus (OV), possesses similar mitotic and vascular permeability effects as VEGF-A_{165} although it shows about 25% homology with mammalian VEGF [35].

VEGF-A_{165} possesses several biological functions and it is produced by many different cell types, including endothelial cells and vascular smooth muscle cells, upon activation by various stimuli including hypoxia, oxidative stress, hormones and other growth factors [49-54]. In angiogenesis, it is the key cytokine and plays a role throughout the process. VEGF-A_{165} mediates vasodilation through nitric oxide (NO), prostacyclin (PGI_2), and PAF synthesis which also modulates vascular permeability [55,56]. NO synthesis is mediated by the activation of nitric oxide synthase (NOS). Our

laboratory has recently demonstrated that the activation of endothelial NOS (eNOS) by VEGF-A_{165} may occur through two pathways: a rapid, calcium-dependent one or a delayed, Akt-dependent one [57]. The resulting vasodilation permits the induction of morphological changes required to enhance the proliferative and mitogenic effects of VEGF-A_{165} [58]. The breakdown of the basal membrane by the localized release of MMPs from endothelial cells is also mediated by VEGF-A_{165} [59] as is the subsequent cell proliferation, migration, and capillary-like tubule formation [60-62]. VEGF-A_{165} is also an inflammatory mediator [63,64] through its translocation and expression of various cell adhesion molecules such as P-selectin, E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) which favour the binding of leukocytes such as neutrophils and monocytes to the endothelium [65,66]. The mitogenic effects of VEGF-A_{165} are mediated by the activation of the p42/44 MAPK intracellular signalling pathway through a Ras-independent (favouring PLC and PKC) or a Ras-dependent pathway requiring scaffolding proteins such as Sos, Grb2, and Shc leading to the transcription [67]. The ability of VEGF-A_{165} to mediate the migration of endothelial cells is due to a rapid interaction with VEGF receptor-2 (VEGFR-2) and integrins and the activation of the p38 MAPK pathway. VEGF-A_{165} is also a cell survival factor through the expression of anti-apoptotic molecules such as Bcl-2, A1, survivin, and XIAP [68,69]. VEGF-A_{165}-mediated cell survival depends on the activation of the PI3K/Akt pathway which may lead to NO synthesis or inhibition of p38 MAPK-mediated apoptotic mechanisms [69-71].

1.3.1.1 Regulation of VEGF-A_{165} expression

The expression of VEGF-A_{165} is primarily induced under hypoxic conditions via hypoxia inducible factor (HIF) regulated elements of the VEGF gene [72]. The HIF-1 complex is composed of two subunits, HIF-1α and HIF-1β [73,74]. Under normal conditions (ie. when oxygen is present), HIF-1β is readily present within the cell whereas HIF-1α is absent since it is rapidly degraded by the ubiquitin-proteosome system by direct proline hydroxylation [75]. This hydroxylation permits the recruitment of the Von Hippel Lindau tumour suppressor gene product (pVHL) which targets HIF-1α for proteosomal degradation [76,77]. Constitutive degradation of hypoxia inducible factor (HIF)-1α is blocked in hypoxia because of the oxygen requirement of HIF-specific proline hydroxylases, promoting the stabilization of HIF-1α and its heterodimerization with HIF-1β, also called the aryl hydrocarbon nuclear translocator (ARNT). These complexes then bind hypoxia-responsive elements (HREs) in the promoters of hypoxia inducible genes and initiate transcription of approximately seventy genes, including genes involved in glucose transport, glycolysis, and angiogenesis [72,78]. Regulation of the expression of VEGF-A_{165} through oxygen levels may also occur in collaboration with other growth factors such as epidermal growth factor (EGF), transforming growth factor (TGF-α and -β), FGF, and platelet-derived growth factor and locally expressed inflammatory cytokines, namely IL-1α, IL-1β and IL-6) [11,28].

1.3.1.2 VEGF receptors (VEGFR)

Three VEGF receptors, all members of the receptor tyrosine kinase (RTK) family, have been identified: VEGFR-1, -2, and -3 with molecular weights ranging from 180 to 220 kDa. The expression of the three receptors at the cell surface membrane is in the monomeric form. However, in the presence of the ligand, dimerization of the monomers occurs through disulfide bonds forming homo- (R1/R1, R2/R2, or R3/R3) or heterodimers (R1/R2 or R2/R3) [79-81]. VEGFR-1 and -2 were initially identified as characteristic to endothelial cells [82,83] but studies have recently observed VEGFR-1 to be present in monocytes, macrophages, and trophoblasts whereas VEGFR-2 is also expressed on hematopoietic stem cells, mesangial cells, and platelets [84]. VEGFR-3 is expressed in most embryonic endothelial cells [85] and primarily in the lymphatic system in adults [45]. Neuropilin-1 (NRP-1), a 140 kDa membrane protein originally identified as a participant in neuronal growth [86], is also extensively expressed in endothelial cells and can interact with VEGFR-2 and potentiate the effects of VEGF-A_{165} [65,80,87,88]. A summary of the VEGF receptors and their respective ligands appears in Figure 3.

VEGF-A_{165} can bind to VEGFR-1 and R-2 but not R-3. VEGFR-1 (fms-like tyrosine kinase or Flt-1) is composed of seven extracellular immunoglobulin (Ig) homology domains, a single transmembrane region and an intracellular tyrosine kinase (TK) domain that is interrupted by a kinase-insert domain [89]. VEGFR-1 was the first receptor identified and its VEGF-A_{165} binding site is located within the second Ig domain [90]. A soluble form of VEGFR-1 (sFlt-1) exists and has neither the seventh

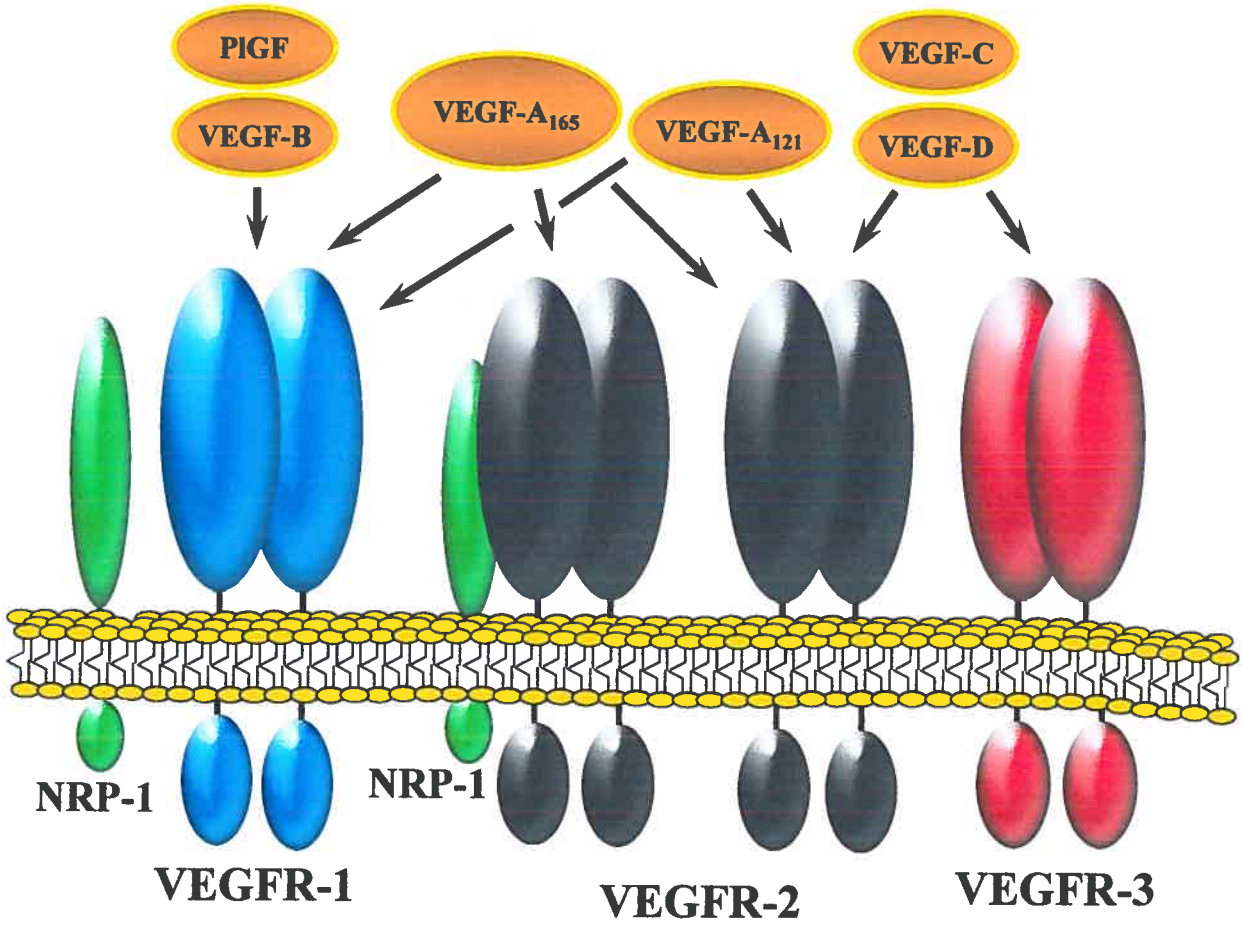

Figure 3: Members of the VEGF family and their respective receptors. PlGF and VEGF-B bind to VEGFR-1 whereas VEGF-C and VEGF-D bind to VEGFR-3. VEGF-A_{165}, the most potent member of the family can bind to VEGFR-1, VEGFR-2, and the VEGFR-2/NRP-1 complex, whereas VEGF-A_{121}, which lacks exon 7, can bind to VEGFR-1 and VEGFR-2 but not NRP-1.

Ig domain, transmembrane domain, nor the kinase domain. Since VEGF-A_{165} binds to this soluble form of VEGFR-1 with great affinity, sFlt-1 sequesters VEGF-A_{165} and inhibits its various effects. Hypoxia increases VEGFR-1 expression, just like VEGF-A_{165} through HIF-1α [91]. In addition to binding VEGF-A_{165}, VEGFR-1 may also bind PlGF and VEGF-B [92,93]. Binding of VEGF-A_{165} elicits only a weak autophosphorylation and it was long believed that VEGFR-1 was a decoy receptor [94,95]. The role of VEGFR-1 in response to VEGF-A_{165} binding remains unclear [96-98]. The inactivation of the VEGFR-1 kinase domain (VEGFR-1 $TK^{-/-}$) in mice embryos produced viable animals with no apparent vascular defects [99]. These observations supported the suggestion that VEGFR-1 was in fact a clearance receptor capable of negatively regulating the mitogenic effects of VEGF-A_{165} by preventing its binding to VEGFR-2 [92]. In mice deficient for the gene coding for VEGFR-1 (VEGFR-$1^{-/-}$), endothelial cells migrate and proliferate normally but the three-dimensional arrangement of the vasculature is impaired and death occurs between embryonic days 8.5 and 9.5 (E8.5 and E9.5) [100]. In other instances, the kinase activity of VEGFR-1 is required, namely for the migration of monocytes and the release of plasminogen (tPA and uPA) and matrix metalloprotease-9 (MMP-9) following treatment with VEGF-A_{165} [101]. We have demonstrated *in vitro* that a selective stimulation of VEGFR-1 by PlGF only marginally induced endothelial PAF synthesis [95] and an intermediate endothelial P-selectin translocation [65]. Furthermore, we reported that *in vivo*, blocking the expression of VEGFR-1 reduced VEGF-A_{165}-mediated angiogenesis by 85% [102]. We have also recently reported that VEGF-A_{165}-induced prostacyclin (PGI_2) synthesis requires the activation of VEGFR-1 and -2 heterodimer [80]. Indeed,

pretreatment with antisense oligomers targeting VEGFR-1 or R-2 mRNA reduced PGI_2 release mediated by VEGF-A_{165} and $-A_{121}$ by up to 79% [80]. Based on the aforementioned evidence, it would therefore appear that VEGFR-1 is involved in selective biological activities mediated by VEGF-A_{165}.

Although the affinity of VEGF-A_{165} for VEGFR-1 is 10 times greater for than for VEGFR-2, the latter is the primary mediator of VEGF-A_{165}-induced angiogenic activity. The overall structure of VEGFR-2 (kinase-insert domain receptor, KDR/fetal liver kinase or Flk-1) is similar to that of VEGFR-1 [103,104]. Genetic inactivation of VEGFR-2 (VEGFR-$2^{-/-}$) is lethal between embryonic days 8.5 (E8.5) and 9.5 (E9.5) [105]. In presence of its ligand, dimerization of VEGFR-2 occurs which leads to transphosphorylation of the receptor and the autophosphorylation of tyrosine residues in the kinase domain [106]. The affinity of the Src homology -2 (SH2) domain for certain tyrosine residues favors the interaction of the receptor with phospholipase C-γ (PLC-γ) [107]. In addition to a robust phosphorylation of VEGFR-2, VEGF-A_{165} activates several intracellular signalling pathways (Figure 4). The chemotactic properties of VEGF-A_{165} towards endothelial cells depend on the activation of the p38 MAPK, an intracellular molecule responsible for the rearrangement of the cytoskeleton [108]. Cellular proliferation is under the control of the p42/44 MAPK pathway through the activation of nuclear transcription factors [108,109]. Activation of PLC-γ and the subsequent hydrolysis of phosphatidylinositol 4,5 diphosphate (PIP_2) into diacylglycerol (DAG) and inositol

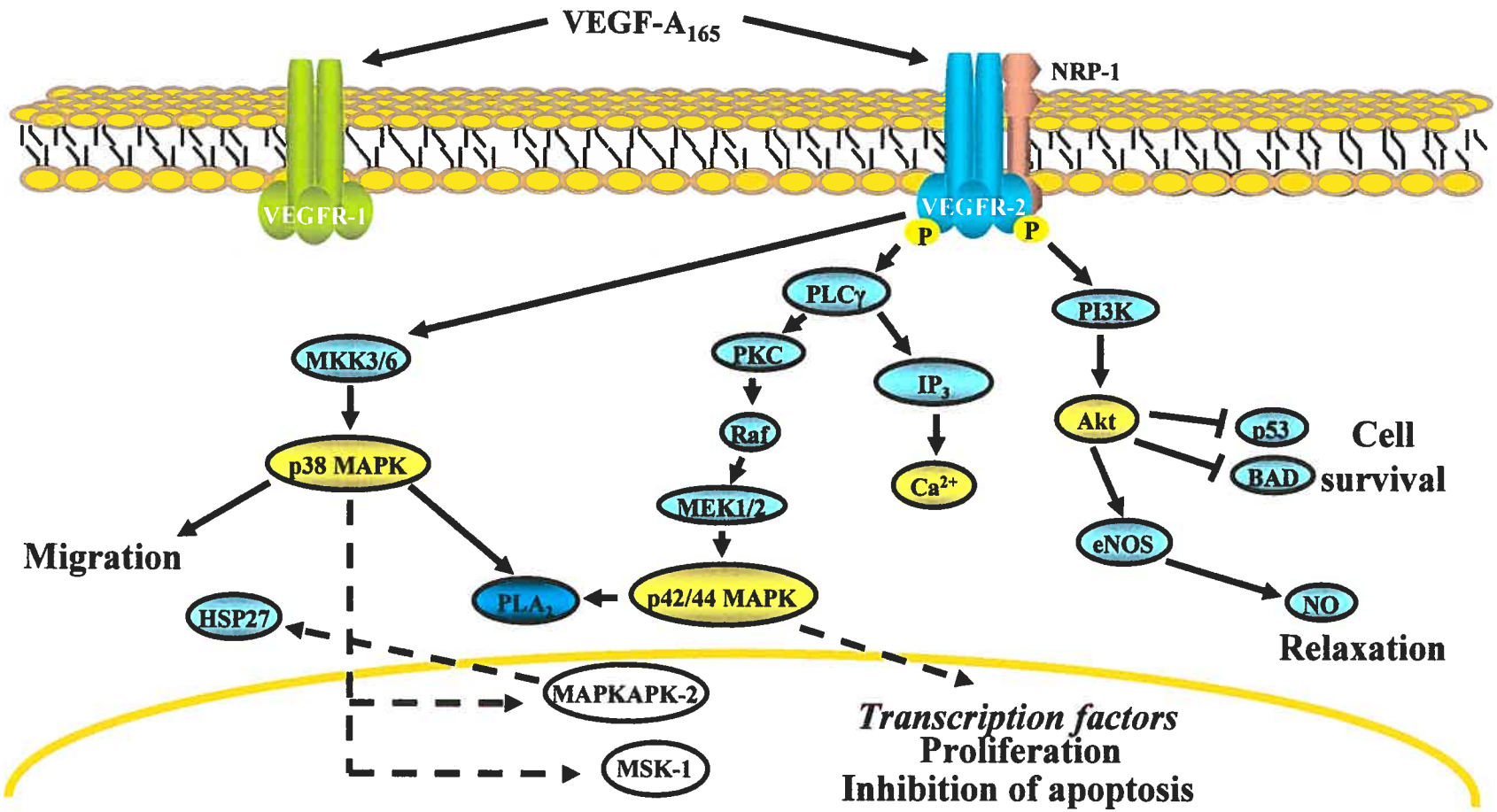

Figure 4: Intracellular signalling pathways activated by VEGF-A_{165}. Stimulation of VEGFR-2 with VEGF-A_{165} activates the p38 MAPK pathway which is responsible for VEGF-A_{165} chemotactic effects. Activation of the p42/44 MAPK pathway regulates VEGF-A_{165}-mediated mitogenic effects whereas PI3K/Akt activation is responsible for cellular survival.

1,4,5 triphosphate (IP_3), an important regulator of intracellular calcium, also resultsin the activation of VEGFR-2 by VEGF-A_{165} [67,108]. The anti-apoptotic effects of VEGF-A_{165} are mediated by the activation of the PI3K/Akt pathway [110]. This pathway is also capable of activating NO synthase (NOS) which is responsible for the VEGF-A_{165}-mediated NO synthesis [70,111].

The interaction between NRP-1 and VEGFR-2 markedly increases VEGF-A_{165}-mediated phosphorylation of VEGFR-2 and cell migration [87]. Initially, NRP-1 was shown to participate in axonal growth through its ability to bind semaphorins and collapsins [86]. Subsequently, NRP-1 was described as capable of binding VEGF-A_{165} but not VEGF-A_{121}, which lacks exon 7 and thus cannot bind NRP-1 [112]. Strongly expressed in endothelial cells, NRP-1, when coexpressed with VEGFR-2, potentiates the binding of VEGF-A_{165} and the ensuing biological effects [87]. Genetic inactivation (NRP-$1^{-/-}$) results in severe defects in vascular development and overexpression caused excess formation of dilated capillaries and blood vessels resulting in embryonic death between E10.5 and E12.5 [86,113]. Together, these data confirm the important role of neuropilin-1 in supporting VEGF-A_{165}-mediated angiogenesis. We have recently reported that NRP-1 increases the migration and proliferation of endothelial cells, platelet-activating factor (PAF) synthesis [88], endothelial P-selectin translocation and adhesion of neutrophils onto endothelial cells [65], and prostacyclin (PGI_2) synthesis [80] induced by VEGF-A_{165}.

1.3.2 Angiopoietins and the Tie2 receptor

In light of its critical role in angiogenesis, VEGF must nonetheless work in concert with other factors to fine-tune vessel formation and regression. Indeed, early therapeutic efforts based on the delivery of a single growth factor to favour angiogenesis resulted in the formation of leaky and dysfunctional vessels [114]. Recently discovered, the angiopoietins, namely Ang1 and Ang2, appear to be important partners for VEGF-A_{165}. Angiopoietins were discovered as ligands for the tyrosine kinase with immunoglobulin and epidermal growth factor homology domains (Tie) receptor family [24,25] selectively expressed within the vascular endothelium (and in other types such as haematopoietic cells) [115-118].

1.3.2.1 The angiopoietins

The angiopoietin family of ligands is comprised of four members, numbered 1 to 4, with similar molecular structures containing a fibrinogen-like domain which binds to Tie2, a coiled-coil domain necessary for dimerization of angiopoietin monomers, and a N-terminal that favors the formation of oligomeric structures essential to the activation of Tie2 (Figure 5) [119-122]. Ang1 was isolated by screening conditioned media from cell lines for specific binding affinity for Tie2 [24] and Ang2 was isolated by screening a cDNA library using Ang1 cDNA as a probe [25]. Ang1 and Ang2 bind to the Tie2 receptor with similar specificity and affinity [24,25]. Ang1

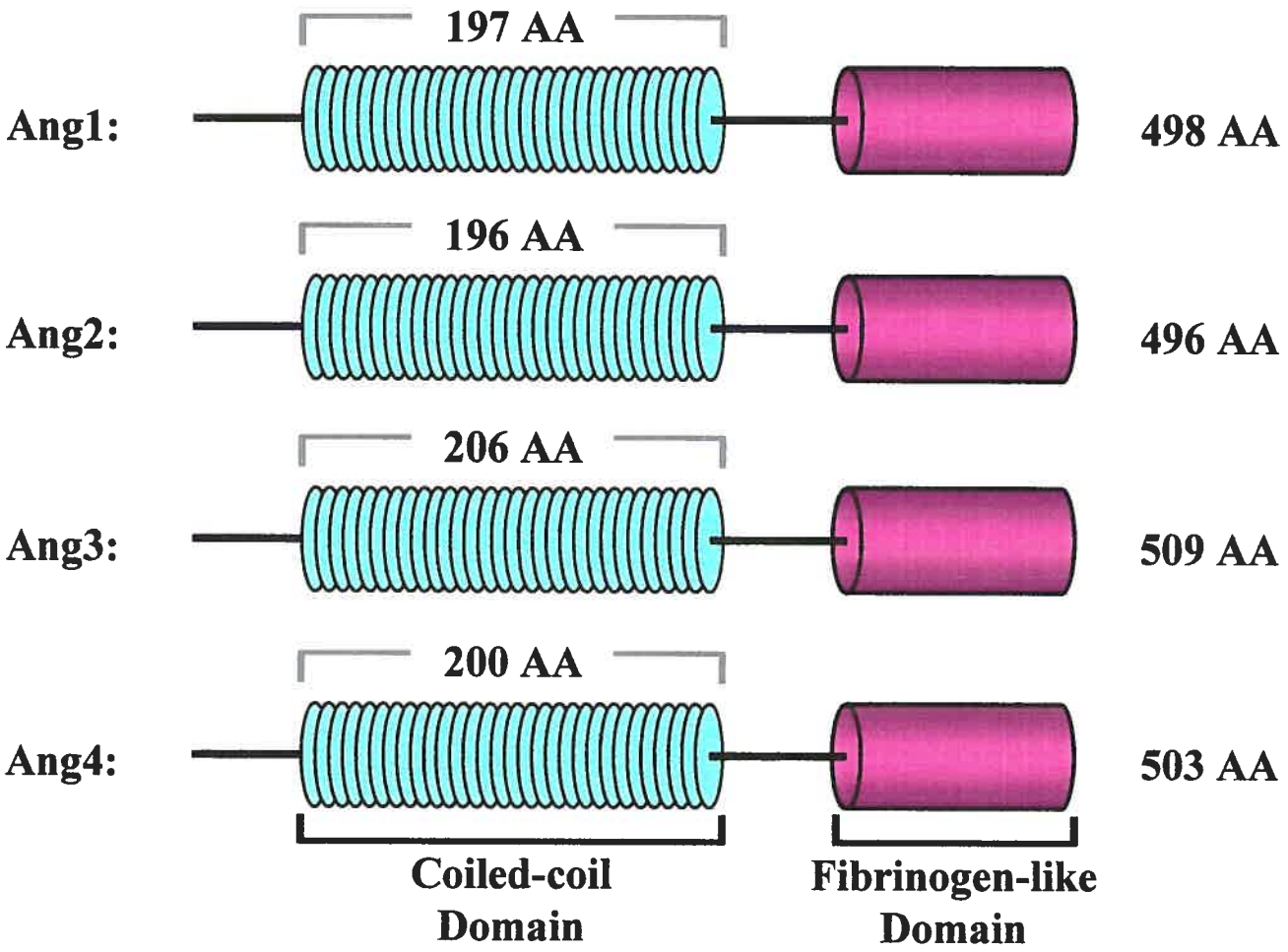

Figure 5: The angiopoietin family. These structurally similar Tie2 receptor ligands possess a coiled-coil domain and a fibrinogen-like domain. (Adapted from Koh *et al.* Exp Mol Med., 2002; 34: 1-11.)

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} [123]. 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 [25]. However, recent findings have shown that Ang2 may, under certain circumstances (concentration, autocrine or paracrine stimulation, duration of treatment, and endothelial cell type) induce Tie2 phosphorylation and biological activities such as EC migration, and *in vitro* tubule capillary-like formation [124-128]. Angiopoietin-3 (mouse) and -4 (human) are interspecies orthologs identified through low stringency hybridization screening using Ang1 and Ang2 cDNA [121]. Ang4 is reported to be a Tie2 agonist whereas Ang3 inhibits Tie2 activation by Ang1. However, as with Ang2, agonist activities for Ang3 have recently been reported [129] although marginal effects have been observed in human endothelial cells.

1.3.2.2 Tie receptors

Identified in the early 1990s, the Tie receptors are a family of receptor tyrosine kinases (RTK) containing two members, Tie1 and Tie2. These two receptors share a unique extracellular amino-terminal domain consisting of three epidermal growth factor (EGF)-like domains, two Ig-like domains flanking the EGF-like repeats, and three fibronectin-type III repeats located just above the transmembrane region (Figure 6). The intracellular domain of the Tie receptors includes two conserved tyrosine kinase domains [116,130,131]. Although ligands for Tie2 are well

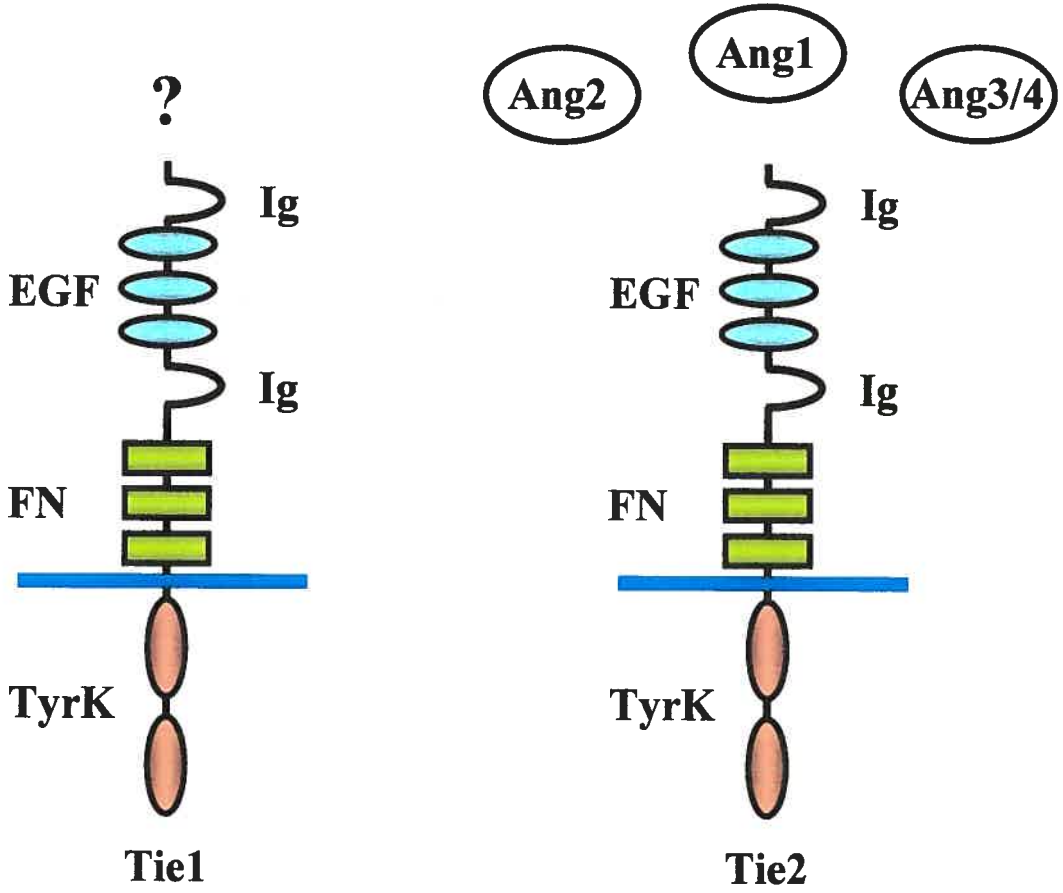

Figure 6: The Tie receptors and their respective ligands. The extracellular region of the Tie receptors consists of immunoglobulin (Ig)-like domains, epidermal growth factor (EGF)-like cysteine repeats, and fibronectin type III (FN) homology domains. The intracellular region of Tie receptors contains the tyrosine kinase (TyrK) domains. Tie1 remains an orphan receptor whereas Tie2 can bind Ang1, Ang2, Ang3, or Ang4. (Adapted from Fiedler *et al.* J Biol Chem, 2003; 3: 1721-27.)

documented, ligands for Tie1 have yet to be identified and thus, Tie1 is considered an orphan receptor. However, evidence is growing that Tie receptors can heterotypically associate through their intracellular domains, suggesting that Tie1 may modulate Tie2 signalling [132]. Furthermore, Tie1 has been shown to undergo proteolytic cleavage by VEGF-A_{165}, releasing a soluble extracellular domain capable of forming a complex with Tie2 [132-134]. A recent study has reported that a chimeric form of Tie1 signals in the absence of Tie2 and activates the PI3K/Akt signalling pathway [135].

1.3.2.3 Role of angiopoietins and the Tie receptors in angiogenesis

Important observations on the biological significance of angiopoietins and the Tie receptors have been made through the use of transgenic and knockout mice. Table 1 presents a summary of the various studies dissecting the role of the Tie receptors and the angiopoietins. Embryos lacking Tie2 or Ang1 die *in utero* by embryonic days 10.5 (E10.5) and 12.5 (E12.5), respectively and display similar phenotypes [136,137]. Both types of embryos display retarded cardiac growth [136,137], and the primary capillary plexus fails to differentiate resulting in a vasculature of low complexity. Furthermore, the existing vessels appear dilated and display disrupted connection of endothelial cells to the extracellular matrix [136,137]. These results suggest that Ang1 recruits and sustains periendothelial support cells and is needed for the maturation of blood vessels during embryonic development. In comparison, the deletion of a single allele of VEGF-A_{165} is sufficient to induce lethality at E11.5 and these embryos show severe defects in their vascular networks [138,139].

Gene	Lethality	Cause of Lethality	Reference
Tie 1 K/O	E13.5 - P1	Poor vessel integrity; edema and hemorrhage	Puri *et al* . 1995
Tie 2 K/O	E10.5	Defective vessel remodeling, organization, and sprouting; heart trabeculation defects; lack of pericyte recruitment; simplification of vessel branching; heart defects	Dumont *et al* . 1994 Sato *et al* . 1995 Patan *et al* . 1998 Puri *et al* . 2001
Tie 2 Transgenic	n/a	Epidermal hyperproliferation; inflammatory cell accumulation; altered dermal angiogenesis; psoriatic-like skin phenotype	Voskas *et al* . 2001
Ang1 K/O	E12.5	Defective vessel remodeling, organization, and sprouting; heart trabeculation defects	Davis *et al* . 1996 Suri *et al* . 1996
Ang1 (K14) Transgenic	n/a	Larger, more branched vessels; resistant to VEGF- induced leakage	Suri *et al* . 1998 Thurston *et al* . 1999
Ang2 K/O	P14	Lymphatic drainage problems; poor vessel integrity; edema; hemorrhage	Gale (personal communication)
Ang2 Transgenic	E9.5 - E10.5	Endocardial-myocardial separation; absence of vessels	Maisonpierre *et al* . 1997

Table 1: Summary of the experiments conducted in transgenic or knockout mice to determine the respective roles of Tie2, Ang1, and Ang2 in angiogenesis.

Interestingly, one can gain insight into the chronology of vessel formation by comparing the phenotype of Tie 2 (Tie2 $^{-/-}$) and VEGFR-2 (VEGFR-2 $^{-/-}$) knockouts. Deletion of the gene coding for VEGFR-2 results in lethality between E8.5 and E9.5 and these embryos are characterized by an absence of blood islands and primary capillary plexi which illustrates the critical role of VEGFR-2 signalling in vascular development [105].

Embryos lacking Tie1 die between E13.5 and birth, with prominent edema in various internal organs, resulting in haemorrhages due to impaired endothelial integrity in the microcirculation [136]. Although not lethal during embryonic development, Ang2 knockout mice rarely exceed 14 days of postnatal life due in part to impaired lymphatic organization and angiogenic defects [126]. In these mice, the lymphatic defects may be resolved upon gene replacement with cDNA encoding for Ang1, suggesting that Ang2 behaves as a Tie2 agonist in lymphatic tissue [126].

Transgenic mice overexpressing Ang1 under the control of the human keratinocyte promoter (K14-Ang1) present a hypervascularization characterized by an increased number, branching, number of endothelial cells, and diameter of dermal blood vessels [123,137]. As with K14-Ang1 mice, K14-VEGF-A_{164} (VEGF-A_{164} in mice is the equivalent of VEGF-A_{165} in humans) mice appear normal and show an increased redness of the skin due to the hypervascularization [123,137]. However, analysis of the phenotype of these animals shows an increased vascular permeability, leukocyte infiltration, and the dermal blood vessels resemble capillaries [123,137]. Interestingly,

mice overexpressing Ang1 and VEGF-A_{164} display a markedly increased dermal vascularization characterized by greater redness, vessels are more numerous and possess characteristics of both phenotypes although no leukocyte infiltration was observed [123,137]. In addition, mice overexpressing Ang2 display a phenotype similar to what was described for $Ang1^{-/-}$ animals. Indeed, overexpression of Ang2 induces lethality between E9 and E10 due to a discontinuous vascular network devoid of capillary branching as well as cardiac malformations [25]. Taken together, these observations suggest that Ang1 inhibits certain inflammatory actions of VEGF-A_{165} and stabilizes the vasculature whereas Ang2 behaves like a Tie2 antagonist during embryonic development (Figure 7).

Although Ang1 does not stimulate endothelial cell proliferation [24], it can nonetheless induce endothelial cell migration [140], tubule formation [141] and sprouting [142,143], and survival from a variety of apoptotic insults [144-146] *in vitro*. Taken together, these observations and the results of the above animal studies demonstrate that Ang1 is a potent proangiogenic factor. Ang1 can also interact directly with integrins at the surface of endothelial cells to favour their adhesion and, in the event of angiogenesis, their migration through its chemotactic effects [140,147]. In addition, prolonged treatment with Ang1 reduces VEGF-A_{165}-mediated neutrophil adhesion and the expression of certain adhesion molecules (ICAM-1, VCAM-1, and E-selectin) [148].

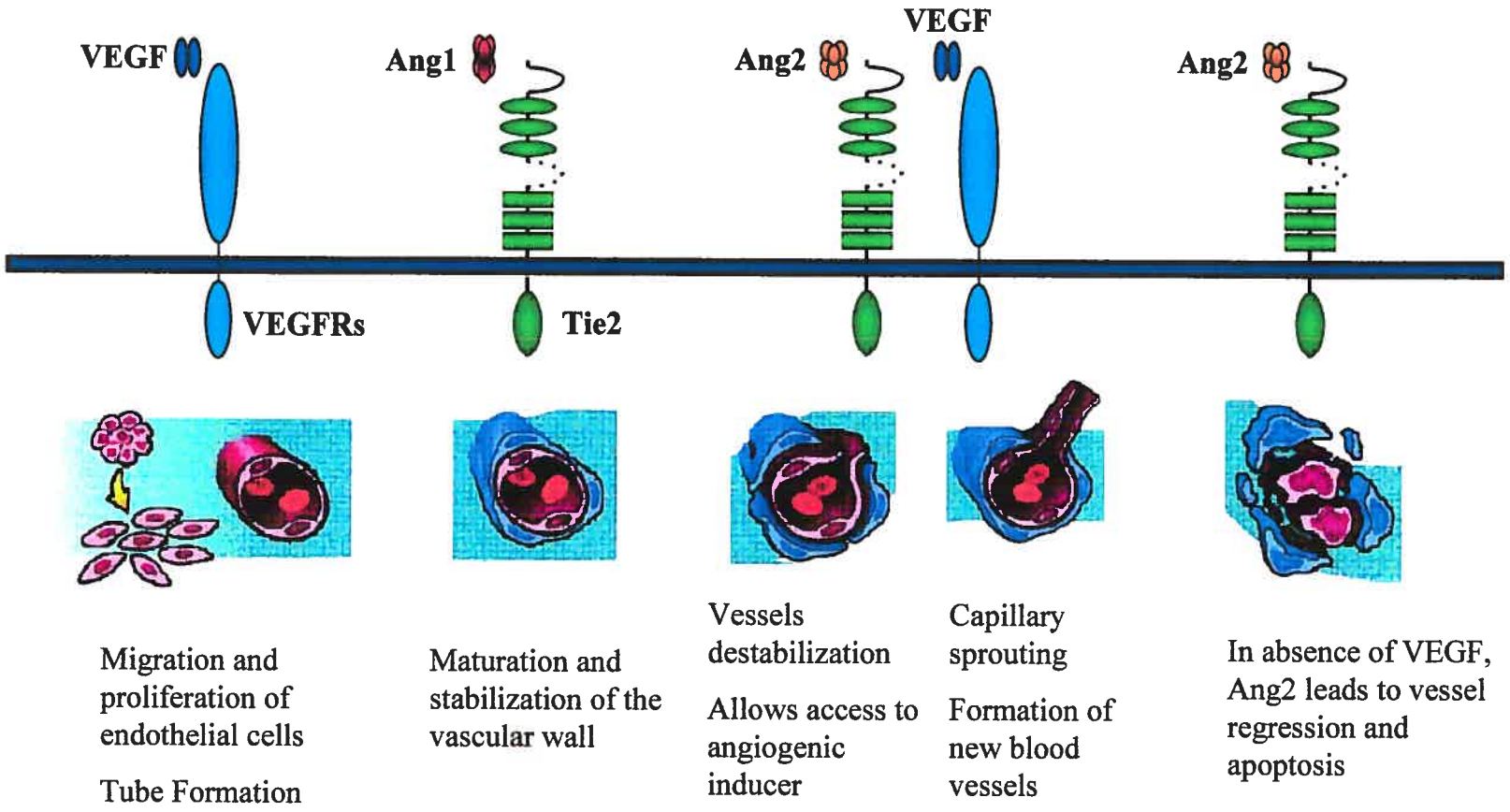

Figure 7: Interaction between VEGF and angiopoietins during angiogenesis. (Adapted from Fam *et al.* Circulation. 2003; 108: 2613-2618.)

The role of Ang2 in blood vessel regulation, on the other hand, is quite complex. The expression of Ang2 in endothelial cells suggests that Ang2 may act in an autocrine manner to control endothelial cell quiescence and responsiveness. As mentioned above, transgenic overexpression of Ang2 is lethal, suggesting that it behaves as a Tie2 antagonist [25]. Initially, *in vitro* experiments confirmed this by demonstrating that Ang2 prevented Ang1-mediated Tie2 phosphorylation [25] and migration [140]. However, Ang2 was shown to activate ecotopically-expressed Tie2 on fibroblasts [25]. Furthermore, recent studies have demonstrated that under certain circumstances, Ang2 may induce, following Tie2 activation, biological activities such as endothelial cell (EC) migration, neutrophil activation, vascular permeability, and *in vitro* tubule capillary-like formation [124,125,127,149,150]. Ang2 also promotes endothelial cell survival through activation of the PI3K/Akt signalling pathway [127]. In addition, HUVEC and fibroblasts plated on various surfaces coated with Ang2 were shown to adhere and spread. In the presence of VEGF, Ang2 induces the proliferation and migration of endothelial cells and promotes the sprouting of new vessels *in vivo* whereas vessel regression occurs when the activity of endogenous VEGF is inhibited [151]. Ang2 has also recently been shown to be stored in endothelial cells within organelles called Weibel-Palade bodies (WPB) from which it can be rapidly secreted, suggesting that Ang2 may be implicated in other processes besides angiogenesis [152].

1.3.2.4 Regulation of the expression of angiopoietins

Ang1 mRNA is primarily expressed in periendothelial cells (pericytes and vascular smooth muscle cells) [146] whereas Ang2 mRNA is selectively expressed in endothelial cells in adult tissues that undergo vascular remodelling such as the ovaries, uterus, and placenta [25].

In the adult, Ang1 is expressed in many tissues such as the reproductive system, skeletal muscle, and small quantities in the heart and liver. A recent study reports that hypoxia modulates the expression levels of Ang1 in pericytes but not in endothelial cells [153]. The presence of a hypoxia-responsive element (HRE) on the Ang1 promoter has yet to be determined. This upregulation may also be mediated in collaboration with other growth factors, namely VEGF-A_{165} which can double Ang1 mRNA expression in pericytes [153]. However, TNF-α has been shown, depending on the study and cell type used to either increase or decrease Ang1 mRNA expression levels [154,155].

Hypoxia also increases Ang2 expression in macro- and microvascular endothelial cells [156,157]. Furthermore, VEGF-A_{165}, bFGF, and TNF-α increase Ang2 mRNA expression in endothelial cells [156-158]. In a rat ovary model of angiogenesis, Ang2 and VEGF mRNA were coexpressed at the front of invading sprouts during active angiogenesis whereas Ang2, but not VEGF, was upregulated during vessel regression [25]. Studies show that tyrosine kinase, MAPK, and PKC-dependent pathways play a crucial role in VEGF-induced Ang2 expression [157]. TNF-α increases Ang2

expression in part through NF-κB activation in human umbilical vein endothelial cells (HUVEC) [155]. It has also been demonstrated that angiotensin II induces Ang2 expression but not Ang1 in cardiac and microvascular cells [159,160]. High levels of Ang2 are expressed in a wide variety of highly vascular tumours ([161] for review) suggesting that Ang2 plays an important role in the initiation of tumour angiogenesis.

1.3.2.5 Signalling pathways activated by angiopoietins

Previous studies reported that Ang1 is capable of activating p38 and p42/44 MAPKs [162] as well as the PI3K/Akt signal transduction pathways [146]. Prior to our study, little was known with regards to potential intracellular events following the activation of Tie2 by Ang2. A summary of the signalling pathways activated upon angiopoietin binding to Tie2 appears in Figure 8. In addition, other studies have reported that Tie2 activates signal transducers and activators of transcription-3 and -5 (STATs) [163] known to play a role in cell proliferation, differentiation, migration, and survival.

Phosphorylated Tie2 interacts cytoplasmically with the Grb family of scaffolding proteins and the p85 subunit of PI3K via their SH2 domains [164]. The interaction of p85 and Tie2 results in the activation of PI3K and the subsequent activation of Akt [165]. Ang1 is implicated in cell survival [144,145] and this activitiy is mediated by the PI3K/Akt pathway [146,166]. Furthermore, the activation of p42/44 MAPK signalling by Ang1 regulates apoptosis in endothelial cells through the phosphorylation of transcription factors favouring the inhibitory action of caspases -

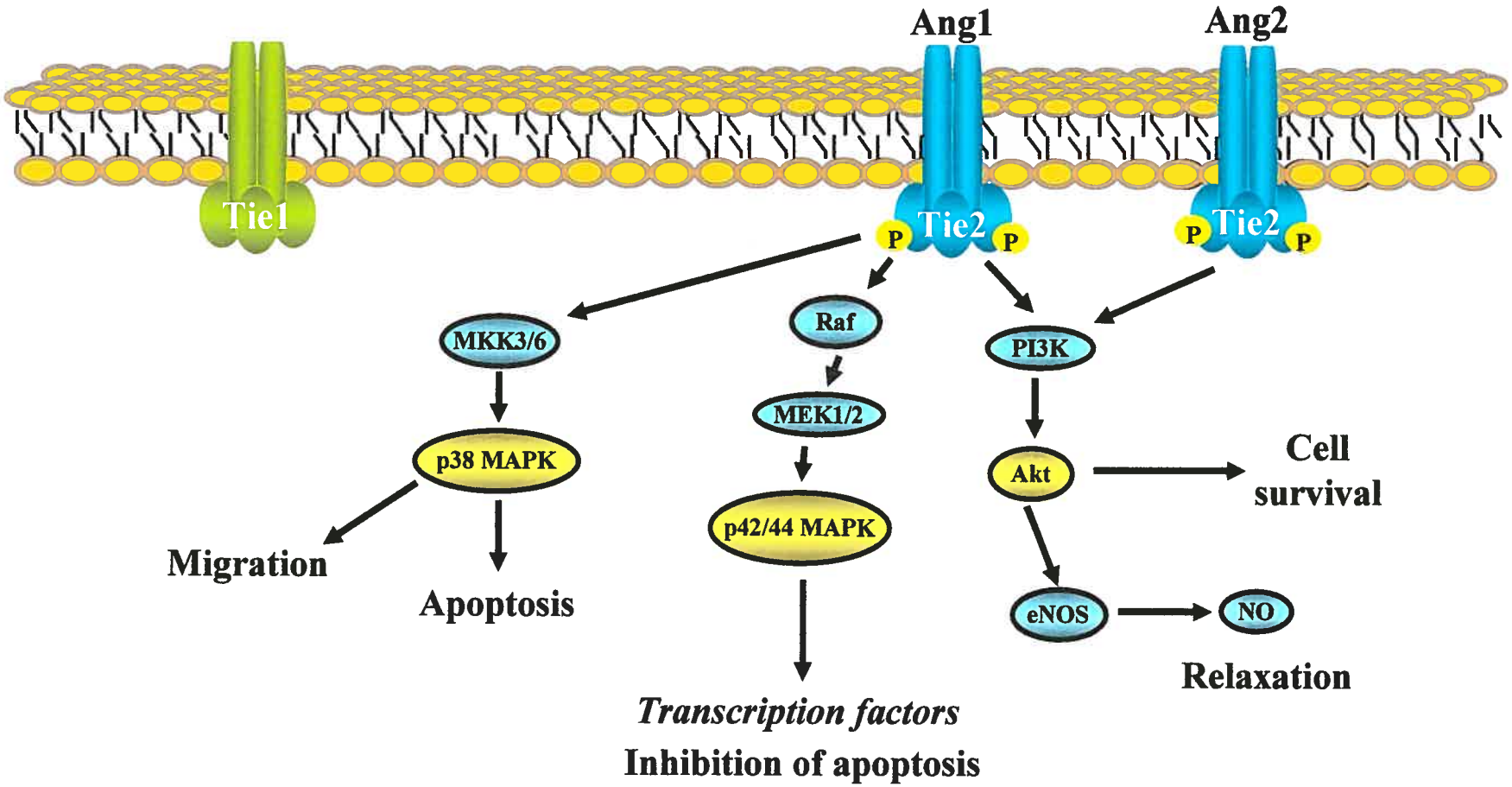

Figure 8: Intracellular signalling pathways activated by the angiopoietins. Stimulation of Tie2 with Ang1 activates the p38 MAPK, p42/44 MAPK, and PI3K/Akt pathways. Ang2 has been shown to activate the PI3K/Akt pathway which mediates cell survival. Signalling by the Tie1 receptor remains to be described.

3, -7, and -9 [162]. The promotion of cell survival contributes to the quiescence and stability of endothelial cells. Another function of Ang1 is the recruitment of pericytes. This activity requires the activation of the p38 MAPK pathway [167]. Ang2 has also been shown to be a cell survival factor through the activation of the PI3K/Akt signalling pathway [127]. However, little is known with respect to the signalling pathways activated by Ang2 in endothelial cells.

1.3.2.6 Angiopoietins in pathological angiogenesis

Angiogenesis plays an important role in several beneficial biological events such as embryonic development, wound healing and, regulation of the female reproductive cycle. However, angiogenesis is also a component of various pathologies. Recent insight into the angiogenic process has determined that the complex sequence of events leading to the formation of neovessels requires the collaboration of various angiogenic mediators, namely VEGF and the angiopoietins. In addition, recent studies have begun defining roles for the angiopoietins in inflammation. Therefore, it is important to present the contribution of angiopoietins to pathological angiogenesis.

1.3.2.6.1 Angiopoietins in tumour vascularization

In addition to a genetic component, tumour progression and propagation requires the induction of tumour vascularization, termed the 'angiogenic switch' ([168] for review). Induction of the angiogenic switch depends on the balance between anti-angiogenic and pro-angiogenic factors and can be induced at various stages of tumour development. Tumours have lost the appropriate balance between positive and

negative regulatory mechanisms that characterize normal, physiological angiogenesis. The switch begins with perivascular detachment and vessel dilation followed by angiogenic sprouting, new vessel formation and maturation to provide the hypoxic regions of the tumour supplied with oxygen and nutrients [168]. Tumour blood vessels are irregularly shaped, dilated, torturous, may have dead ends, and are often leaky and haemorrhagic due to an overproduction of VEGF. It has been suggested that cancer cells initially encroach upon existing microvessels (co-option) followed by destabilization and regression of the vessels in the center of the tumour, and initiation of new capillary growth at the periphery of the tumour [158]. In certain cases, tumours may recruit endothelial precursor cells from bone marrow which become incorporated into the walls of growing vessels through the action of VEGF-A_{165}, PlGF, and Angl [169,170]. However, the number of circulating endothelial precursors is low and although some tumours have been shown to progress in this manner [171], most tumour neovascularization occurs through angiogenesis.

The role of Angl in tumour angiogenesis is not clear. Overexpression of Angl has been observed in malignant glioblastoma [172,173], neuroblastoma [174], non-small cell lung cancer [175], and variably in other tumours as well [161]. However, certain studies using xenograft models demonstrate ectopic expression of Angl in breast [176] and colon [177] cancer cells results in decreased tumour proliferation and angiogenesis. In addition, a similar tumour inhibitory role for Angl has been observed in squamous cell carcinoma (SCC) models overexpressing Angl [178]. The explanation for this apparent paradox may lie in the ability of Angl to recruit mural cells to stabilize

vessels since vessels in the aforementioned tumours all displayed increased pericyte recruitment, suggesting that mature blood vessels functionally inhibit tumour angiogenesis through enhanced cell-cell junctions, rendering the vessel less prone to angiogenesis [161]. In reviewing the literature, what becomes apparent is that although absolute levels of either Ang1 or Ang2 increase or decrease depending on the type of tumoural model, the ratio of Ang1 to Ang2 within tumours shifts in favour of Ang2 [179]. High levels of Ang2 mRNA are observed in highly vascularized tumours such as glioblastoma, Kaposi's sarcoma, cutaneous angiosarcoma, hemangioma, thyroid tumours, as well as gastric and colorectal cancer [172,180-184]. The overexpression of Ang2 in tumours is often accompanied by that of VEGF [161] suggesting that the destabilization of vessels by Ang2 permits VEGF-mediated angiogenesis to proceed. It would therefore appear that Ang2 plays an important role in the initiation of tumour angiogenesis through its ability to antagonize Ang1-induced vessel stabilization. The ability of tumours to shift the angiogenic balance towards a pro-angiogenic state by altering the balance between angiopoietins may implicate Ang2 as a candidate for the angiogenic switch [179].

1.3.2.6.2 Angiopoietins in inflammation

The role of Ang1 in inflammation is currently under investigation and in light of the animal studies demonstrating the ability of Ang1 to inhibit certain inflammatory actions of VEGF-A_{165}, recent reports indicate that Ang1 possesses anti-inflammatory abilities. Indeed, Ang1 was shown to reduce VEGF-A_{165}-stimulated leukocyte adhesion to HUVEC through a reduction in the expression of adhesion molecules,

namely intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin [148]. In this study, Ang1 by itself had no effect on leukocyte adhesion or the expression of adhesion molecules within 4 hours but it counteracted VEGF-induced inflammation. The antiinflammatory effects of Ang1 were further demonstrated when endothelial permeability, neutrophil adhesion to endothelial cells, and interleukin-8 (IL-8) production by endothelial cells were inhibited following at least 1 hour of treatment with Ang1 [185]. In addition, polymorphonuclear neutrophil (PMN) migration across endothelial monolayers stimulated by TNF-α was also reduced in response to treatment with angiopoietin-1 [186]. Moreover, the localization of proteins such as platelet endothelial cell adhesion molecule-1 (PECAM-1) into junctions between endothelial cells was enhanced as evidenced by a decrease in basal permeability and inhibition of permeability responses to thrombin and vascular endothelial growth factor [186]. VEGF-mediated permeability is associated with the disruption of endothelial cell junctional complexes, dissociation of β-catenin from vascular endothelial (VE)-cadherin, and accumulation of β-catenin in the cytosol [187,188] and this pathway requires the activation of protein kinase C-β (PKCβ) isoforms which is blocked by Ang1[189]. Taken together, the above *in vitro* studies appear to confirm that Ang1 inhibits the proinflammatory effects of VEGF-A_{165}.

Until recently, little was known about the potential role of Ang2 in inflammatory processes. However, Ang2 has been shown *in vivo* to induce edema formation in the mouse paw in a dose-dependent manner which is blocked by administration of

soluble Tie2 receptor or Ang1 [150]. In addition to edema, a weak stimulatory effect on leukocyte migration was observed in the mouse paw following treatment with Ang2 [150]. Similarly, Ang2 injected into the mouse air pouch produced a modest effect on leukocyte migration [150]. The recent identification of Ang2 as a stored, rapidly secreted molecule in endothelial cells strongly suggests that Ang2 may play a role in rapid vascular homeostatic reactions such as inflammation [152]. Stimulation of endothelial cells with thrombin, phorbol myristate acetate (PMA), or histamine induced a rapid release of Ang2 (within 5 minutes) from Weibel-Palade bodies whereas treatment with VEGF-A_{165}, Ang1, bFGF, or transforming growth factor β (TGF-β) did not induce Ang2 release [152]. The released Ang2 could, in turn, modulate the destabilization of the vasculature in the presence of VEGF-A_{165} or act in an independent manner and promote proinflammatory events.

1.4 The inflammatory response and angiogenesis

It is now well established that the inflammatory response and angiogenesis are mutually inclusive based on observations that tumours demonstrate increased vascular permeability and the presence of inflammatory cells such as neutrophils and macrophages at angiogenic sites [8,23,190]. During inflammation, new vessels provide essential oxygen and nutrients to inflamed tissues. Furthermore, the vasculature enables inflammatory cells, namely neutrophils and monocytes/macrophages, to reach the affected site and secrete cytokines, growth factors (ie. VEGF-A_{165}) [191], and proteases (ie. MMP-2 and MMP-9) [192,193] to regulate the angiogenic response.

Inflammation can be divided into two major categories, chronic or acute. Chronic inflammatory diseases such as rheumatoid arthritis, atherosclerosis, and inflammatory bowel disease are characterized by a prolonged duration (weeks to years) and infiltration of monocytes and fibrosis in which active inflammation, tissue destruction, and attempts at tissue repair are occurring simultaneously [194]. Acute inflammation is of relatively short duration (minutes to days) and is characterized by vasodilation which facilitates the local delivery of soluble mediators and inflammatory cells, the exudation of protein-rich plasma, and the migration of neutrophils to the site of injury [194]. The vasodilatory response is mediated by nitric oxide (NO) throught the action of nitric oxide synthase (NOS) [195] and prostaglandins mostly PGI_2 produced from arachidonic acid through the action of cyclooxygenases [196]. The exudation of plasma into the surrounding tissues is termed edema and is representative of an increase in vascular permeability resulting from the actions of inflammatory mediators such as VEGF-A_{165}, histamine, bradykinin, leukotrienes, and PAF which act on intercellular junctions between endothelial cells. The inflamed region swells and displays a characteristic redness and warmth.

1.4.1 PAF, an inflammatory mediator

Initially identified in rabbit leukocytes, platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) has now been shown to possess a variety of biological activities [197]. PAF has been shown to participate in embryogenesis and angiogenesis through its ability to induce endothelial cell migration [198]. Studies have also reported that PAF can act as both an inter- and intracellular messenger [199], can

modulate cardiac function by acting on cardiomyocytes, and reduce blood pressure [200,201]. PAF may also mediate both an acute and chronic inflammatory response [202]. The increase in vascular permeability observed upon treatment with PAF is the result of PAF binding to its extra- and intracellular receptors (PAFR) and inducing an increase in intracellular calcium which in turn favours the contraction of the actin skeleton, shrinkage of the cell and the formation of gaps between adjoining cells [201,203,204]. PAF may also be colocalized at the endothelial cell surface with P-selectin and mediate leukocyte rolling, activation, and adhesion onto the endothelium [205,206].

1.4.1.1 PAF synthesis

PAF may be synthesized through two distict pathways. Physiological amounts of PAF are synthesized via the *de novo* pathway in the kidney and central nervous system [207,208] whereas PAF synthesis in stimulated inflammatory cells is regulated by the 'remodelling pathway'. This pathway rapidly generates large quantities of PAF and regulates 'very early' (2 to 5 minutes) and 'early' (10 to 40 minutes) PAF synthesis and its activity is controlled by cytosolic calcium (Ca^{2+}) and the activation of either a phospholipase A_2 (PLA_2) or a coenzyme A (CoA)-independent transacyclase to generate lyso-PAF, a biologically inactive PAF precursor ([197] for review). Lyso-PAF is in turn converted into PAF by the action of lyso-PAF acetyltransferase (lyso-PAF AT) which mediates the transfer of the acetyl group from acetyl-CoA to the free hydroxyl moiety at the sn-2 position of

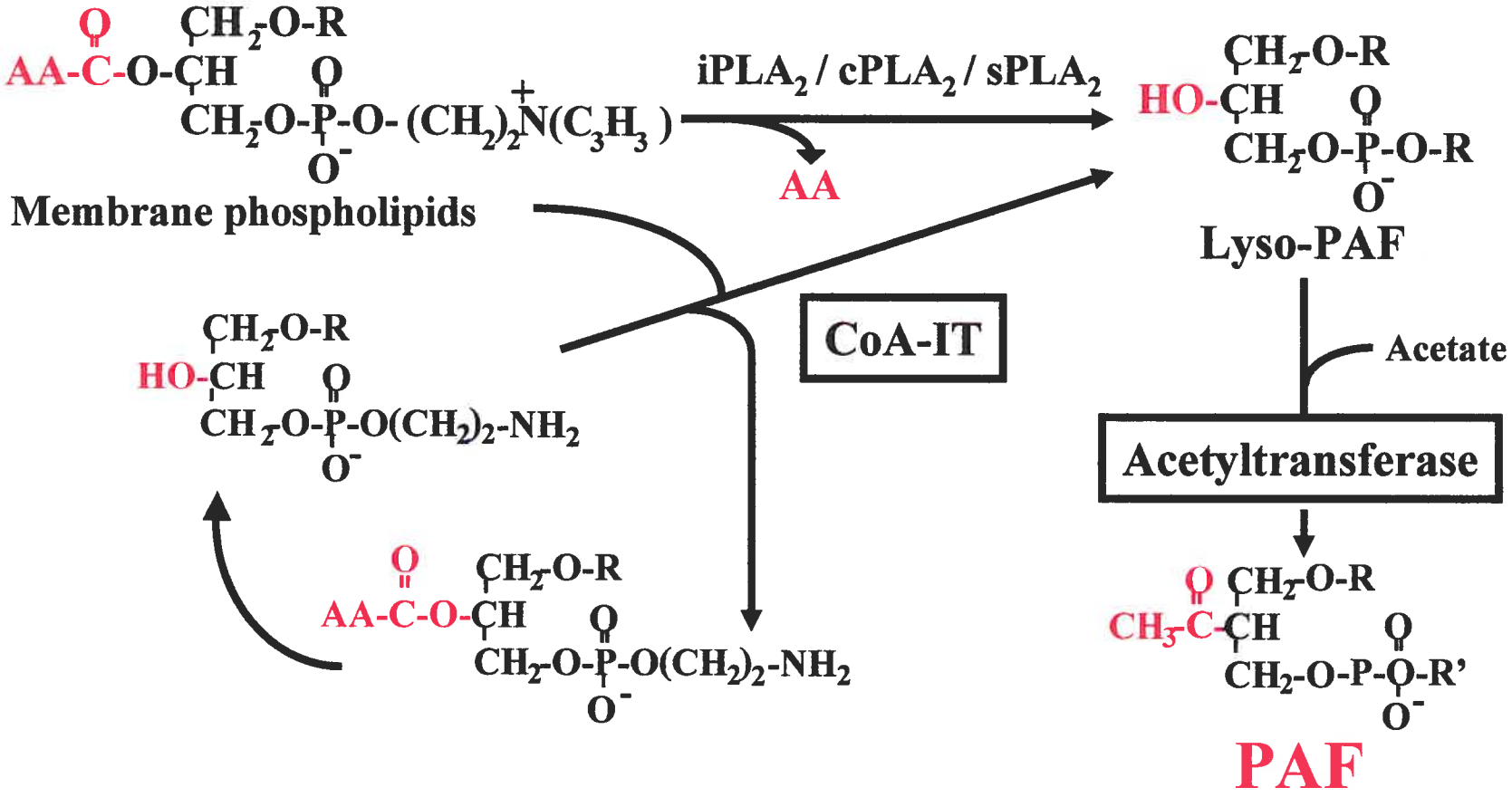

Figure 9: Summary of the remodelling pathway of endothelial PAF synthesis. The hydrolysis of membrane phospholipids into lyso-PAF and arachidonic acid (AA) is catalyzed by a phospholipase A_2 (PLA_2) or a coenzyme A (CoA)- independent transacylase (CoA-IT). Lyso-PAF is in turn converted into PAF by lyso-PAF acetyltransferase. With permission from Dr. P.N. Bernatchez.

lyso-PAF [209,210]. A summary of the 'remodelling pathway' leading to PAF synthesis is presented in Figure 9.

1.4.1.2 Role of PAF in angiogenesis

Growing evidence demonstrates that PAF is directly implicated in angiogenesis. PAF has been shown to mediate endothelial cell migration *in vitro* [198] and budding of new vessels in mice subjected to sustained release of PAF [197,211,212]. The angiogenic potential of PAF is also mediated by its effects on adhesion properties of endothelial cells which enhance leukocyte adherence and ultimately their ability to release angiogenic mediators [213]. The role of PAF in pathological angiogenesis has also been examined. PAF released by certain tumours into the extracellular environment has been reported to act in a paracrine fashion and promote budding of new vessels, increasing the potential malignancy of these tumours [197,214]. Several types of tumours also express PAF receptors (PAFR) [197,215] and in vivo overexpression of PAFR has been shown to favour tumour development [216]. Taken together, these observations demonstrate the role of PAF in tumourigenesis.

The role of PAF in angiogenesis may be linked to VEGF-A_{165}. It has been demonstrated that VEGF-A_{165} increases vascular permeability through the synthesis of PAF in endothelial cells [56]. An increase in vascular permeability is one of the characteristic elements of acute inflammation. In an inflammatory setting, activated neutrophils and other leukocytes release inflammatory mediators such as VEGF and PAF which, besides acting on vascular permeability through the release of

metalloproteases from the endothelium to favour detachment of endothelial cells from the basal membrane [59,217], promote P-selectin translocation to the surface of endothelial cells [65,205] and favour adhesion onto activated endothelial cells as discussed above.

1.4.2 The phospholipase A_2 family

The phospholipase A_2 (PLA_2) superfamily consists of a broad range of enzymes defined by their ability to catalyze the hydrolysis of the middle (*sn-2*) ester bond of substrate phospholipids to liberate arachidonic acid (AA) and lysophospholipids [218-220]. This action is important for the synthesis of eicosanoids such as prostaglandins and leukotrienes from AA precursors and for the synthesis of PAF from phospholipids as described in the previous section. Initially isolated from cobra venom and then from various other sources, PLA_2 are categorized into eleven (11) classes (I – XI) [218,221] and four families: cytosolic ($cPLA_2$), secreted ($sPLA_2$), calcium-independent ($iPLA_2$), and PAF-acetylhydrolases ([220] for review).

Located within the cytosol of resting cells, $cPLA_2$ is a high molecular weight (85 kDa) PLA_2 that shows a marked preference for AA over other fatty acids and requires submicromolar concentrations of Ca^{2+} for activation [222]. Ubiquitously and constitutively expressed in most cells and tissues [223], $cPLA_2$ plays an essential role in the initiation of stimulus-induced AA liberation from phospholipids, supplying cyclooxygenase and lipoxygenases which synthesize eicosanoids. Evidence from studies utilizing cells from $cPLA_2$-null mice show that animals lacking $cPLA_2$ are

unable to generate these lipid mediators [224] In addition, $cPLA_2$ has been shown to mediate PAF synthesis from phospholipids in neutrophils [225]. Activation of $cPLA_2$ is tightly regulated by cytosolic Ca^{2+} levels and sustained phosphorylation of Ser^{505} by p42/44 or p38 MAPKs [226].

The secretory PLA_2 ($sPLA_2$) family, which contains 10 isozymes, consists of low molecular weight (14-19 kDa), secretory enzymes implicated in various biological processes such as the modification of eicosanoid synthesis, inflammation, host defense, and atherosclerosis [219]. $sPLA_2$ hydrolyze the *sn-2* ester bond of glycerophospholipids in the presence of millimolar Ca^{2+} concentrations with no particular fatty acid specificity [219]. Inflammatory cells such as neutrophils, macrophages, and mast cells store $sPLA_2$-IIA in secretory granules and release it promptly upon cell activation [227]. Large amount of $sPLA_2$-IIA are detected in exudated fluids and plasma of patients and animal models with inflammatory conditions [228,229]. Type V $sPLA_2$ ($sPLA_2$-V) is the primary secreted PLA_2 in the mouse but its levels are also markedly elevated in human diseases such as cardiac dysfunction and inflammation [220]. We have recently reported that VEGF-A_{165}-mediated endothelial PAF synthesis is mediated by $sPLA_2$-V [230].

Fully active in the absence of Ca^{2+}, $iPLA_2$ shows no strict *sn-2* fatty acid preference. The specific contributions of $iPLA_2$ isoforms to biological events have yet to be fully defined although it has been suggested that they partake in phospholipids remodelling through the deacylation of phospholipids [231].

Finally, PAF-AH, also referred to as group VIIA PLA_2, is a 45 kDa secreted protein which hydrolyzes the ester bond in the *sn-2* position of PAF to liberate acetate and its inactive intermediate, lysoPAF [220].

1.4.3 Adhesion of leukocytes to the endothelium

The first step towards the extravasation of circulating inflammatory cells into the extravascular tissues involves the induction of leukocyte rolling on the endothelium. In response to inflammation, adhesion molecules are expressed at the surface of endothelial cells and leukocytes favouring the transient attachment (rolling) of leukocytes to the endothelium. Rolling elicits a priming response which favours the slowing down of circulating leukocytes and their subsequent firm adhesion to endothelial cells. Firm adhesion leads to diapedesis followed by migration into the interstitium towards the site of inflammation or angiogenesis where leukocytes can, in turn, secrete a plethora of inflammatory and angiogenic mediators. The sequence is graphically represented in Figure 10. Three main families of adhesion molecules, each possessing distinct molecular structures, participate in the overlapping steps of the leukocyte adhesion cascade: the selectins, the integrins, and the immunoglobulin (Ig) superfamily.

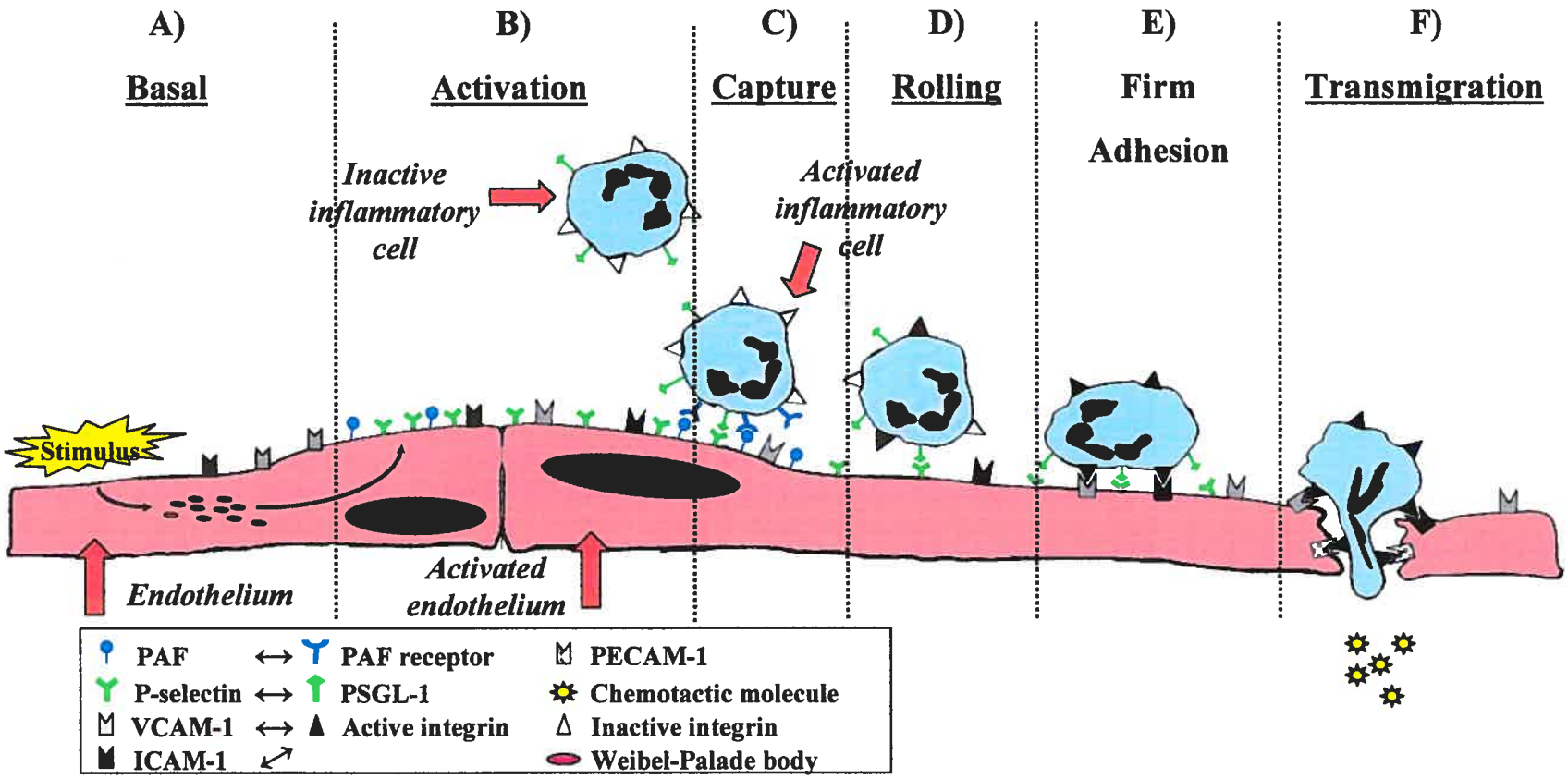

Figure 10: Leukocyte rolling and adhesion. Upon stimulation (A), endothelial cells become activated and express P-selectin and PAF at their surface (B) and are in turn recognized by their counter-receptors present at the surface of circulating leukocytes, mediating the initial capture and rolling of activated leukocytes on activated endothelium (C and D). Firm adhesion is regulated by the binding of integrins, expressed at the surface of activated leukocytes, to ICAM or VCAM on the endothelial surface (E). Transmigration requires the interaction of PECAM and integrins to facilitate the movement of leukocytes through the endothelial layer towards the site of inflammation (F). (Adapted from: Krieglestein *et al.* Am.J. Hypert., 2001; 14: 44S-54S.)

1.4.3.1 The selectins

The selectins play a critical role in the initial capture from the bloodstream and rolling of leukocytes on the vascular endothelium. The selectin family of adhesion molecules consists of three members. The expression of E-selectin is induced upon endothelial cell activation, L-selectin is constitutively expressed on the surface of leukocytes, and P-selectin is stored within the alpha (α) granules of platelets and the Weibel-Palade bodies (WPB) of endothelial cells and translocated to the cell surface membrane upon cell activation.

The selectins are Type 1 transmembrane glycoproteins and possess an extracellular domain composed of a lectin domain, an epidermal growth factor-like (EGF) domain, and short consensus repeats (SCR) also referred to as complement binding (CB) elements. The lectin domain is responsible for ligand recognition and binding whereas the EGF domain stabilizes the conformation of the lectin domain [232]. Truncating the number of SCR has been shown to impair the efficiency with which P-selectin could support rolling of leukocytes suggesting that these domains are important in extending P-selectin a sufficient length from the plasma membrane [233]. Moreover, the number of SCR varies from two (2) in L-selectin to nine (9) in P-selectin and accounts for the size variation amongst the three selectins [234]. All three selectins are anchored in the membrane by a single transmembrane region followed by a short cytoplasmic tail [234,235] as presented in Figure 11.

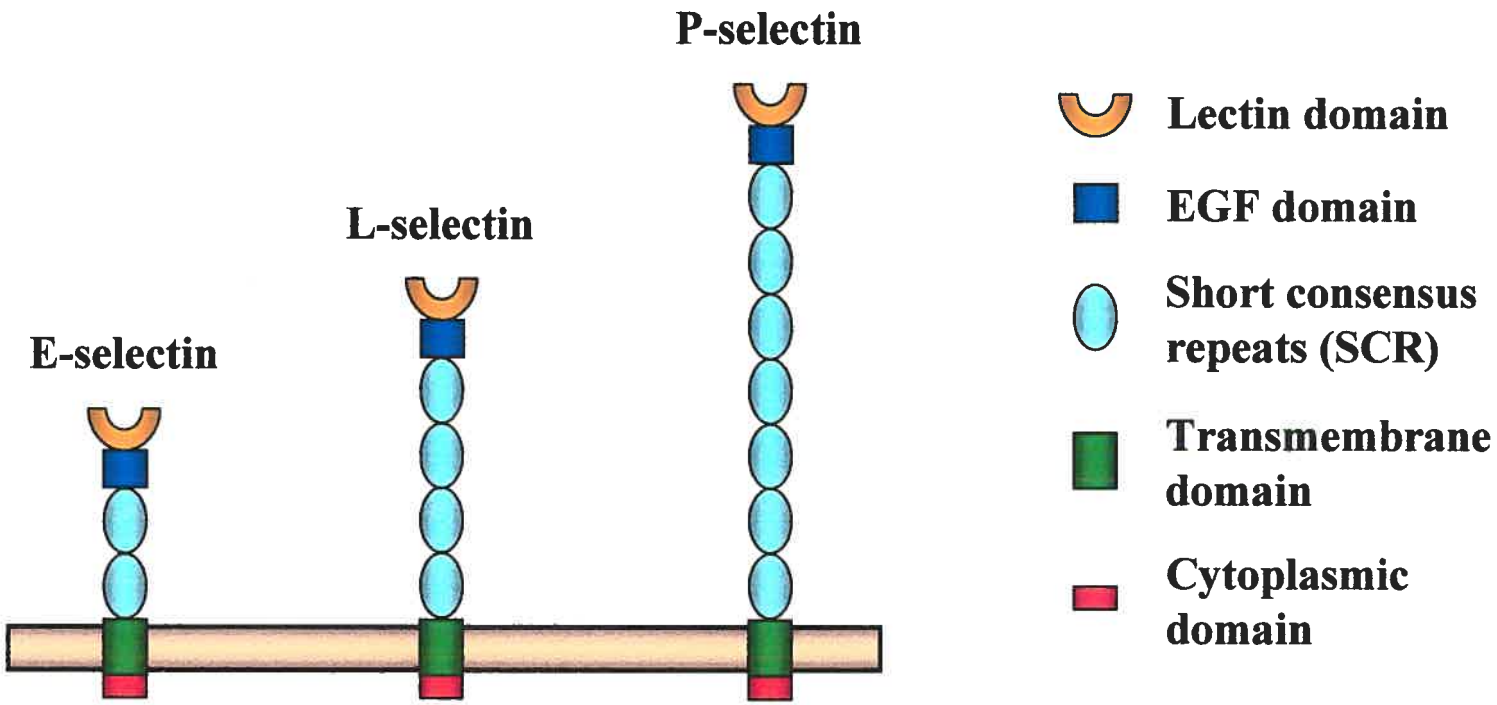

Figure 11: Structural organization of selectins. Selectins are composed of an NH_2-terminal lectin domain, a single epidermal growth factor (EGF)-type repeat, and various numbers of short consensus repeats (SCR) also referred to as complement binding domains. Selectins have a single transmembrane domain and a short cytoplasmic tail. (Adapted from Vestweber and Blanks, Physiol Rev., 1999; 79: 181-213.)

E-selectin is only expressed at the surface of activated endothelial cells in response to inflammatory mediators such as lippopolysaccharide (LPS) or interleukin (IL)-1β [235,236] and induction achieved through transcription with 3 to 4 hours [237]. E-selectin is implicated in the rolling of leukocytes primarily during transition from rolling to firm adhesion [238] through interactions with its ligands present at the surface of leukocytes, E-selectin ligand-1 (ESL-1) [239] and P-selectin glycoprotein ligand-1 (PSGL-1) [240].

L-selectin is constitutively expressed on the surface of leukocytes [241] and has been shown to slow rolling leukocytes to allow for their activation by mediators such as IL-8 and PAF [242]. In addition, activation of leukocytes results in the rapid downregulation of L-selectin within minutes [243] by proteolytic activity through the action of metalloprotease cleaving L-selectin at an extracellular site proximal to the cell membrane [243,244]. This phenomenon, termed L-selectin shedding facilitates detachment of leukocytes from the endothelial cells as they start migrating through the endothelial cell layer [234] and is indicative or leukocyte activation. Certain studies have suggested intracellular signalling resulting from the binding of L-selectin to its ligands, expressed at the surface of endothelial cells, increases β2 integrin-mediated adhesion [242,245,246].

In endothelial cells, P-selectin is stored within Weibel-Palade bodies, translocated to the cell surface upon activation by inflammatory mediators such as histamine, thrombin and phorbol esters, or hypoxia [247]. Following translocation, P-selectin may be recycled back into the cell by endocytosis [248] although it has recently been

suggested that P-selectin may be cleaved and a portion of the P-selectin measured in the plasma may be of endothelial origin [249,250]. Endothelial P-selectin translocation is rapid and transient, maximal within 10 minutes and returning to baseline by 3 hours [251-253] although additional synthesis may be brought about within 2 hours by cytokines such as IL-1, tumour necrosis factor-α (TNF-α), and by LPS or thrombin [252-254]. P-selectin binds to P-selectin glycoprotein ligand-1 (PSGL-1) located on the surface of leukocytes. Studies using knockout mice deficient for the gene coding for PSGL-1 have demonstrated that leukocyte rolling is defective in these animals [255-258].

1.4.3.2 The Integrins

The steps following leukocyte rolling govern the firm adhesion of inflammatory cells to the endothelium, their movement through the inter-endothelial junctions and their migration into the inflamed region. The integrins located on the surface of leukocytes play a critical role in this series of events.

Structurally, the integrins are type I transmembrane glycoproteins comprised of non-covalently associated alpha (α) and beta (β) subunits. The integrins are a large family, 19 alpha subunits and 8 beta subunits have been identified comprising 25 known integrins [259]. Leukocytes express the β_2 integrin (CD18) subfamily in association with α_L (α_L β_2, CD11a/CD18, or LFA-1) and α_M (α_M β_2, CD11b/CD18, or Mac-1) subunits [260,261] and these integrins play an important role in the firm adhesion of leukocytes to endothelial cells. The primary ligand for the β_2 integrins is intercellular adhesion molecule-1 (ICAM-1) present at the surface of activated

endothelial cells. LFA-1 may also bind to ICAM-2 and -3, albeit with a lower affinity than to ICAM-1 [262] whereas Mac-1 binds to a wide variety of ligands [263]. The integrins may be activated by inflammatory mediators such as PAF and leukotriene B_4 (LTB_4) and chemotactic agents like IL-8 binding to their respective G-protein-coupled receptors (GPCR) on the surface of leukocytes. Signalling pathways associated with GPCR act on the cytoplasmic tail of β_2 integrins and through a conformational change, increase their affinity for their ligand [264]. Within seconds of activation by chemokine receptor binding or tethering on E- or P-selectins, CD18 expression and affinity is increased on leukocytes [264-266].

1.4.3.3 The Ig superfamily

Completion of the process of leukocyte recruitment follows the development of strong interactions between integrins and Ig-superfamily members [267]. Ig-superfamily members are characterized by their extracellular Ig domain analogous to the immunoglobulin light and heavy chains. Certain members of this family, namely ICAM-1 and -2 expressed on endothelial cells are counter-receptors to leukocyte-borne integrins. ICAM-1, which possesses 5 Ig domains, is only weakly expressed on the surface of endothelial cells under normal conditions [268]. However, its expression is greatly increased in the presence of IL-1β, TNF-α, and LPS [269-271]. ICAM-2 has only 2 Ig domains and contrary to ICAM-1, it is constitutively expressed on the endothelium and its expression level is not affected by cytokines [272]. Diapedesis also requires the action of platelet endothelial adhesion molecule-1 (PECAM-1) which is expressed along the borders of endothelial cells [273].

1.5 Rationale

Over the course of the last several years, work conducted in our laboratory has centered on the inflammatory role of VEGF-A_{165} in angiogenesis. Indeed, our laboratory has delineated the role of VEGFR-2 and the cell signalling pathways implicated in VEGF-A_{165}-mediated endothelial PAF synthesis [95,230,274]. In addition, we have recently demonstrated the implication of endogenous PAF in the VEGF-A_{165}-mediated translocation of P-selectin and the adhesion of neutrophils onto activated HUVEC [65]. Since angiopoietins act in concert with VEGF-A_{165} to modulate vascular plasticity during postnatal neovascularization [275], we therefore sought to investigate the role of these recently characterized angiogenic factors in mediating proinflammatory events.

1.6 Purpose

Based on previous observations with regards to VEGF-A_{165} and on the capacity of angiopoietins to regulate vascular integrity, we sought to investigate whether Ang1 and/or Ang2 could modulate endothelial PAF synthesis and if so, to define the intracellular signalling pathways. Furthermore, we recently demonstrated that Ang1 and Ang2 are also both capable of promoting endothelial P-selectin translocation and neutrophil adhesion onto endothelial cells [149]. Thus, we also sought to dissect the cellular mechanisms implicated in angiopoietin-mediated endothelial P-selectin translocation and assess the role of PAF in this process. Together, these two objectives would allow us to delineate the role of angiopoietins in acute inflammation.

2.0 Article #1

Ms. Ref. No.: CLS-D-05-00080R2
Title: ANGIOPOIETINS -1 AND -2 ARE BOTH CAPABLE OF MEDIATING ENDOTHELIAL PAF SYNTHESIS: INTRACELLULAR SIGNALLING PATHWAYS
Cellular Signalling

Dear Martin,

I am pleased to confirm that your paper "ANGIOPOIETINS -1 AND -2 ARE BOTH CAPABLE OF MEDIATING
ENDOTHELIAL PAF SYNTHESIS: INTRACELLULAR SIGNALLING PATHWAYS" has been accepted for publication in Cellular Signalling.

Comments from the Editor and Reviewers can be found below.

Thank you for submitting your work to this journal.

With kind regards,

CLS EDITORIAL OFFICE
Editorial Office
Cellular Signalling

Comments from the Editors and Reviewers:

Dear Dr Sirois

Your manuscript entitled 'ANGIOPOIETINS -1 AND -2 ARE BOTH CAPABLE OF MEDIATING ENDOTHELIAL PAF SYNTHESIS: INTRACELLULAR SIGNALLING PATHWAYS' has now been formally accepted for publication in Cellular Signalling. You will next hear from Elsevier Publishing regarding proofs etc. Thank you for submitting this most interesting paper to the journal.

Yours sincerely,

Professor NJ Pyne

Identification des auteurs

1. **Identification**
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2. **Description de l'article**
   Titre: *Angiopoietin -1 and -2 are both capable of mediating endothelial PAF synthesis: intracellular signalling pathways.*

   Liste des auteurs: Ricardo Maliba, Stéphanie Lapointe, Paul-Eduard Neagoe, Alexandre Brkovic, et Martin G. Sirois.

   Soumission à la revue: *Cellular Signalling*

3. **Déclaration de tous les coauteurs autres que l'étudiant**
   À titre de coauteur de l'article identifié ci-dessus, je suis d'accord pour que Ricardo Maliba inclus cet article dans sa thèse de doctorat qui a pour titre *Regulation of the proinflammatory properties of angiopoietins*.

Stéphanie Lapointe — [redacted] — 6 janvier 2006
Coauteur — Signature — Date

Paul-Eduard Neagoe — [redacted] — 6 janvier 2006
Coauteur — Signature — Date

Alexandre Brkovic — [redacted] — 6 janvier 2006
Coauteur — Signature — Date

Martin G. Sirois — [redacted] — 6 janvier 2006
Coauteur — Signature — Date

ANGIOPOIETINS -1 AND -2 ARE BOTH CAPABLE OF MEDIATING ENDOTHELIAL PAF SYNTHESIS: INTRACELLULAR SIGNALING PATHWAYS

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Running Title: Ang1 and Ang2 are proinflammatory mediators

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ANGIOPOIETINS -1 AND -2 ARE BOTH CAPABLE OF MEDIATING ENDOTHELIAL PAF SYNTHESIS: INTRACELLULAR SIGNALLING PATHWAYS

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2.1 SUMMARY

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 hours (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.

Key words: angiopoietins, Tie2 receptor, platelet-activating factor, inflammation

2.2 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, monocytes/macrophages and neutrophils 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, 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.

2.3 MATERIAL & METHODS

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].

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 minutes 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 hours. 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 minutes 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 the 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, 1:1 000 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 (1:1 000 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].

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 minutes 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 (1:1 000 dilution; New England Biolabs). Membranes were

subsequently stripped and reprobed to visualize corresponding total protein expression.

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 minutes. 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 minutes prior to stimulation with Ang1 or Ang2 (1 nM) for 240 minutes or VEGF-A_{165} for 15 minutes. 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 ß-counter. The authenticity of synthesized PAF was confirmed by an identical elution pattern to standard [^{3}H]-PAF (New England Nuclear) [3, 14].

VEGF ELISA

The content of 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.

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 minutes) in serum-free DMEM. Following stimulation, the cells were rinsed and fixed with a 1% paraformaldehyde-PBS solution for 20 minutes. 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 minutes. Glass coverslips were mounted using 1,4-diazabicyclo-2-2-2octane (DABCO/glycerol (1:1) solution. BAEC were observed on a Zeiss Axiovert 100M 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 nm X 143 nm X 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 files format series. Transparent projections were produced using the projection tool of the LSM 510 software. VEGF levels at the cell surface membrane was assessed by quantifying the summation of voxels 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.

Statistical analysis

Data are mean $\pm$ 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.

2.4 RESULTS

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 minutes, and corresponding to a 21-fold increase over PBS-treated cells (Figure 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 minutes (Figure 1B).

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 (Figure 2). In our study, stimulation of BAEC with Ang1 (1 nM) activates p42/44 MAPK and Akt in a time-dependent manner (Figure 2A and E) with maximal effects at 7.5 minutes maintained through 30 minutes of stimulation. Treatment with Ang1 induces a rapid and transient activation of p38 MAPK with a maximal phosphorylation at 7.5 minutes (Figure 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 minutes of stimulation (Figure 2B) but as with Ang1, this activation is maintained 30 minutes post-stimulation. Secondly, activation of PI3K/Akt by Ang2 is delayed compared to Ang1 and not sustained (20 minutes versus 7.5 minutes; Figure 2F). Ang2 activation of p38 MAPK produces a pattern similar to what was observed with Ang1 (Figure 2D). As a positive control, BAEC were also stimulated with VEGF-A_{165} (1 nM; 7.5 minutes).

Regulation of PAF synthesis by Ang1 and Ang2

We previously reported that VEGF-A_{165} induces a rapid and transient (within 15 minutes) 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 minutes), maximal at 4 hours and sustained for at least 6 hours post-treatment (Figure 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 minutes to 30 minutes followed by a "burst" phase culminating at 4 hours. 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 minutes (788% increase over PBS values) and was degraded within 30 minutes, 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 hours) 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 hours (Figure 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 (Figure 3C).

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 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 minutes prior to stimulation with Ang1 or Ang2 (1 nM) for 4 hours. Inhibition of VEGFR-1 and VEGFR-2 with VTK prompted a 51% and 43% decrease in PAF synthesis mediated by Ang1 and Ang2, respectively (Figure 4). When only VEGFR-2 activity was inhibited, Ang1 and Ang2-mediated PAF synthesis was diminished by 42% and 26%, respectively (Figure 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 minutes (Figure 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 minutes prior to stimulation with angiopoietins (1 nM) for 7.5 minutes. 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 minutes to 6 hours, whereas most endogenous VEGF was quantified from BAEC lysates (Figure 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 cell surface membrane was set to 1 (Figure 6A), and was slightly higher than negative control in which PBS-treated cells were incubated with isotypic rabbit IgG instead of primary VEGF IgG (RI = 0.9; Figure 6B). Treatment with Ang1 (1 nM) for 7.5 minutes resulted in a marked redistribution of VEGF on cell surface membrane (RI = 10.2; Figure 6C), which remained noticeable up to 4 hours post-stimulation (RI = 1.97; Figure 6D). Treatment of BAEC with Ang2 (1 nM) induced as well an acute but less intense relocalization of endogenous VEGF within 7.5 minutes (RI = 2.81; Figure 6E), but which was sustained up to 4 hours (RI = 3.56; Figure 6F).

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 minutes (6.3 and 9.2-fold increase) respectively (Figure 7A and B). Treatment with VEGF-A_{165} (1 nM) for 7.5 minutes was used as positive control (Figure 7C).

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 minutes prior to treatment with Ang1 (1 nM) for 4 hours, decreased EC PAF synthesis by 65, 93, 93, and 100%, respectively (Figure 8A). Similarly, Ang2-mediated EC PAF synthesis at 4 hours was reduced by 73% to 100% following pretreatment with the aforementioned inhibitors (Figure 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 concentrations prevented the phosphorylation of p42/44 MAPK, p38 MAPK and PI3K (data not shown).

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 (Figure 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 (Figure 8B). We also treated BAEC with LY311727 (100 μM), an inhibitor of $sPLA_2$-V 15 minutes prior to stimulation with Ang1 or Ang2 (1 nM; 4 hours) and observed a reduction of 43 and 55% in PAF synthesis, respectively (Figure 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).

2.5 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 minutes 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).

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 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 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].

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 hours) 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 minutes), early (10 - 40 minutes), or delayed (4 - 8 hours) [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 hours 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 prevent but even slightly increased EC PAF synthesis by both Ang1 and Ang2 at 4 hours. 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 minutes of stimulation with Ang1 and to a lesser extent with Ang2, and which remained noticeable up to 4 hours post-stimulation with both angiopoietins. Furthermore, we observed that a treatment with Ang1 or Ang2 were both 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 (Figure 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.

2.6 ACKNOWLEDGMENTS

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2.8 FIGURE LEGENDS

FIGURE 1: Activation and expression of Tie2 in BAEC. Confluent BAEC were treated with Ang1 (A), or Ang2 (B) for up to 2 hours. 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 chemiluminesence. 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.

FIGURE 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 minutes. BAEC were also stimulated with VEGF-A_{165} for 7.5 minutes 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.

FIGURE 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 minutes in the presence of [^{3}H]-acetate. As positive control BAEC were treated with VEGF-A_{165} for 15 minutes. Ang1 (A) and Ang2 (B) mediate endothelial PAF synthesis with maximal values observed following 4 hours 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.

FIGURE 4: Pretreatment with VEGF receptor (VEGFR) inhibitors attenuates angiopoietin-mediated endothelial PAF synthesis at 4 hours. 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 minutes prior to stimulation with Ang1 or Ang2 (1 nM) for 4 hours 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.

FIGURE 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 hours. 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.

FIGURE 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 minutes (C and E, respectively) or 4 hours (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.

FIGURE 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 minutes to 4 hours. 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 chemiluminesence. Membranes were subsequently stripped and VEGFR-2 protein

expression was determined following incubation with rabbit polyclonal anti-mouse VEGFR-2 IgG.

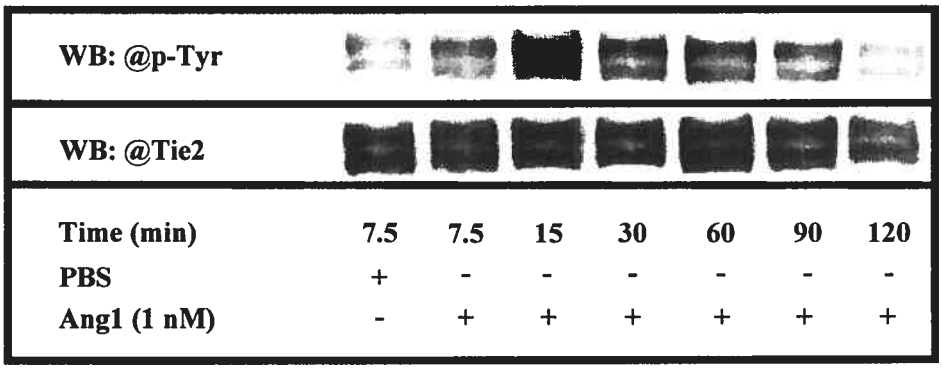
FIGURE 8: Angiopoietin-mediated endothelial PAF synthesis requires the activation of p38 MAPK, p42/44 MAPK, and PI3K/Akt signalling pathways and $sPLA_2$-V. (A) Confluent BAEC were pretreated with either PD98059 (10 μM), SB203580 (10 μM), LY294002 (5 μM), or Wortmannin (500 nM) 15 minutes prior to 4 hours 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 minutes prior to 4 hours 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.

FIGURE 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.

2.9 FIGURES

Figure 1

(A) IP: @Tie2 140 kDa



(B) IP: @Tie2 140 kDa

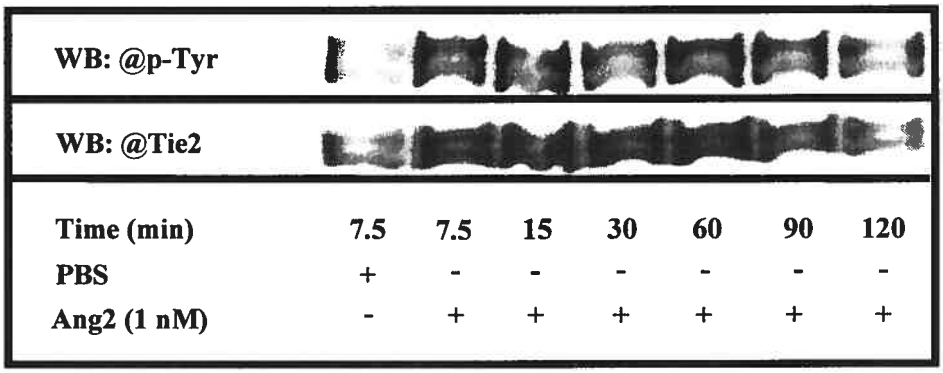


Figure 2

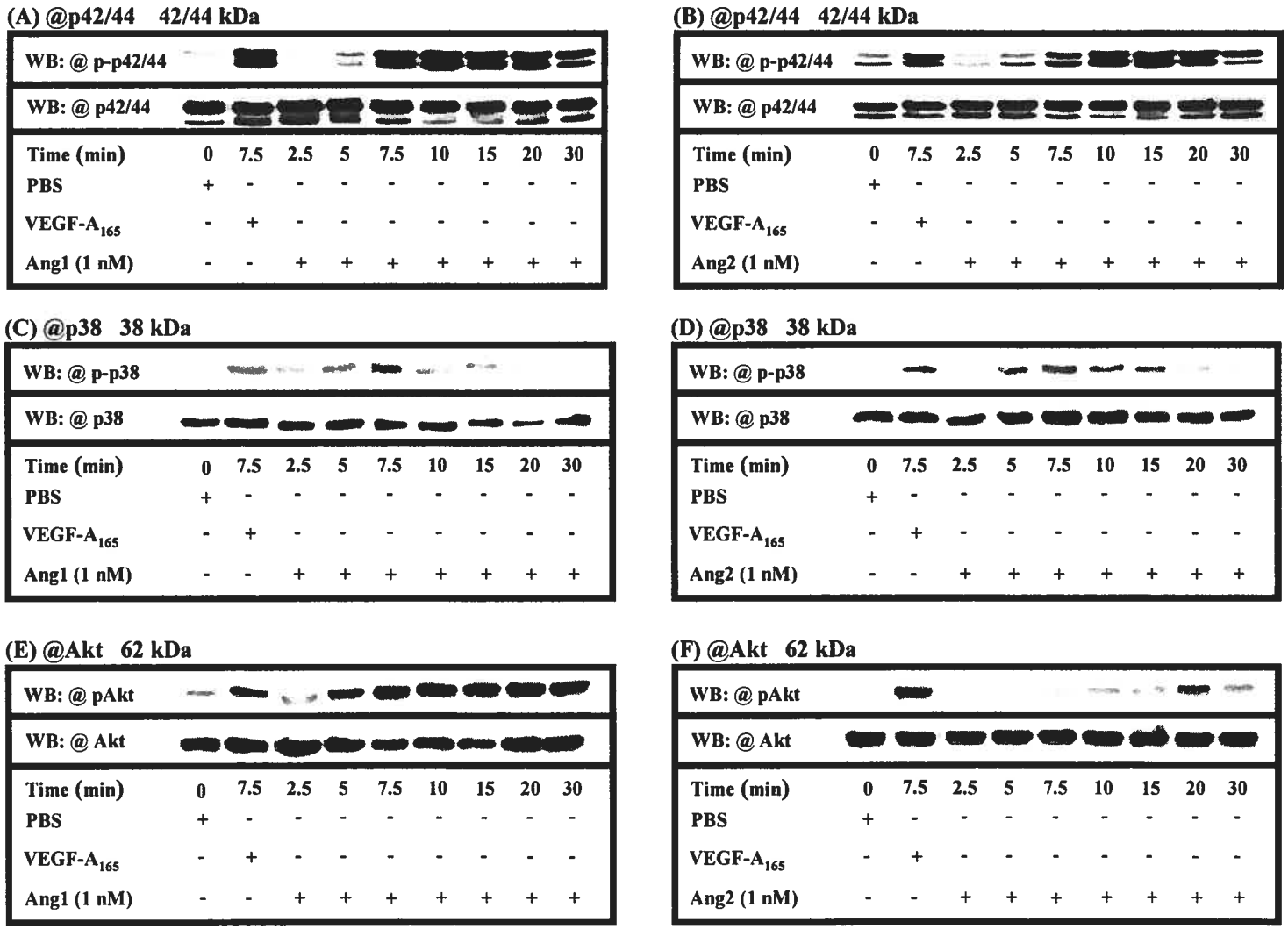

(A) @p42/44 42/44 kDa
WB: @ p-p42/44
WB: @ p42/44
Time (min) 0 7.5 2.5 5 7.5 10 15 20 30
PBS + - - - - - - - -
VEGF-A165 - + - - - - - - -
Ang1 (1 nM) - - + + + + + + +
(B) @p42/44 42/44 kDa
WB: @ p-p42/44
WB: @ p42/44
Time (min) 0 7.5 2.5 5 7.5 10 15 20 30
PBS + - - - - - - - -
VEGF-A165 - + - - - - - - -
Ang2 (1 nM) - - + + + + + + +
(C) @p38 38 kDa
WB: @ p-p38
WB: @ p38
Time (min) 0 7.5 2.5 5 7.5 10 15 20 30
PBS + - - - - - - - -
VEGF-A165 - + - - - - - - -
Ang1 (1 nM) - - + + + + + + +
(D) @p38 38 kDa
WB: @ p-p38
WB: @ p38
Time (min) 0 7.5 2.5 5 7.5 10 15 20 30
PBS + - - - - - - - -
VEGF-A165 - + - - - - - - -
Ang2 (1 nM) - - + + + + + + +
(E) @Akt 62 kDa
WB: @ pAkt
WB: @ Akt
Time (min) 0 7.5 2.5 5 7.5 10 15 20 30
PBS + - - - - - - - -
VEGF-A165 - + - - - - - - -
Ang1 (1 nM) - - + + + + + + +
(F) @Akt 62 kDa
WB: @ pAkt
WB: @ Akt
Time (min) 0 7.5 2.5 5 7.5 10 15 20 30
PBS + - - - - - - - -
VEGF-A165 - + - - - - - - -
Ang2 (1 nM) - - + + + + + + +

Figure 3

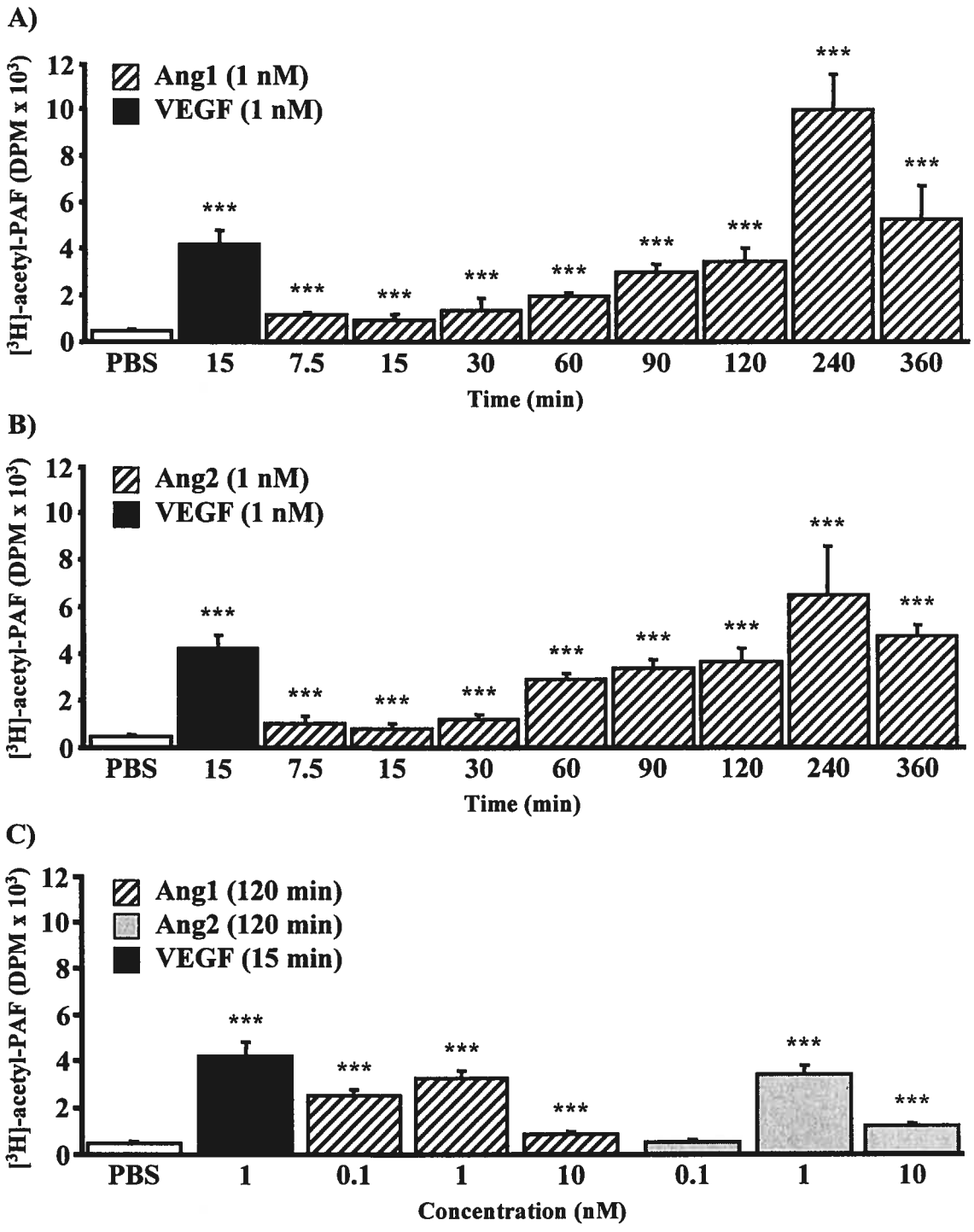

A)
Ang1 (1 nM)
VEGF (1 nM)
[3H]-acetyl-PAF (DPM x 10³)
PBS
15
7.5
15
30
60
90
120
240
360
Time (min)
B)
Ang2 (1 nM)
VEGF (1 nM)
[3H]-acetyl-PAF (DPM x 10³)
PBS
15
7.5
15
30
60
90
120
240
360
Time (min)
C)
Ang1 (120 min)
Ang2 (120 min)
VEGF (15 min)
[3H]-acetyl-PAF (DPM x 10³)
PBS
1
0.1
1
10
0.1
1
10
Concentration (nM)

Figure 4

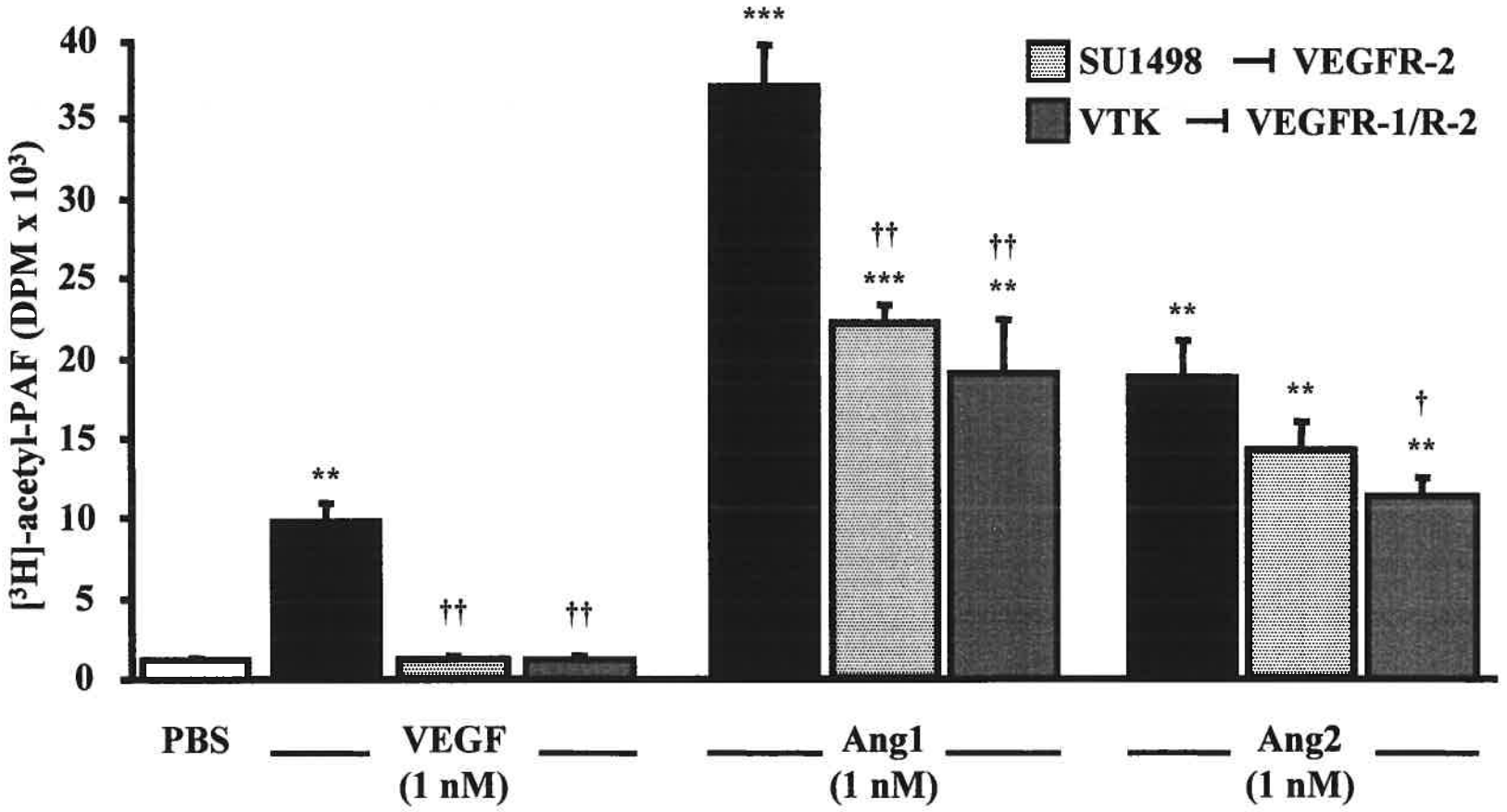

[3H]-acetyl-PAF (DPM x 10³)
40
35
30
25
20
15
10
5
0
SU1498 ⊣ VEGFR-2
VTK ⊣ VEGFR-1/R-2
PBS
VEGF (1 nM)
Ang1 (1 nM)
Ang2 (1 nM)

Figure 5

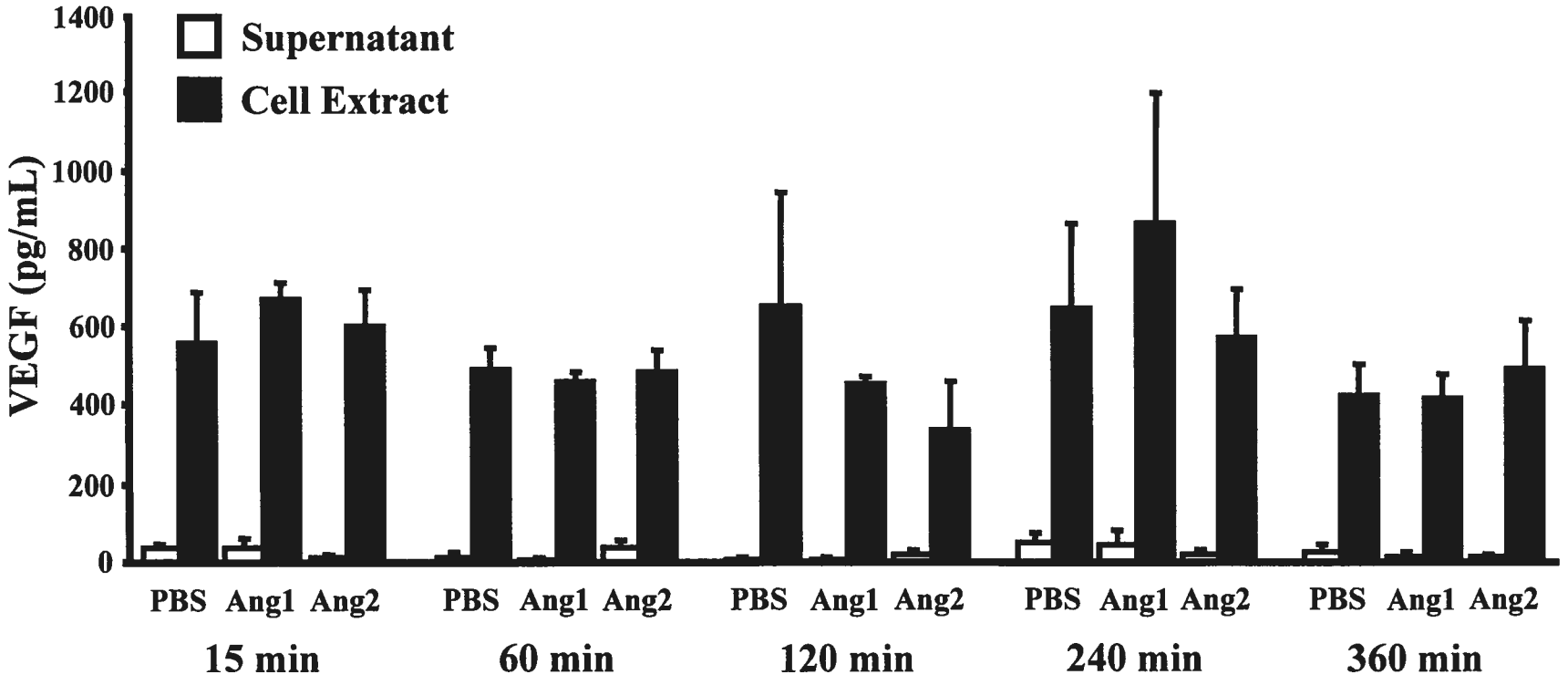

Supernatant
Cell Extract
VEGF (pg/mL)
1400
1200
1000
800
600
400
200
0
PBS Ang1 Ang2
15 min
PBS Ang1 Ang2
60 min
PBS Ang1 Ang2
120 min
PBS Ang1 Ang2
240 min
PBS Ang1 Ang2
360 min

Figure 6

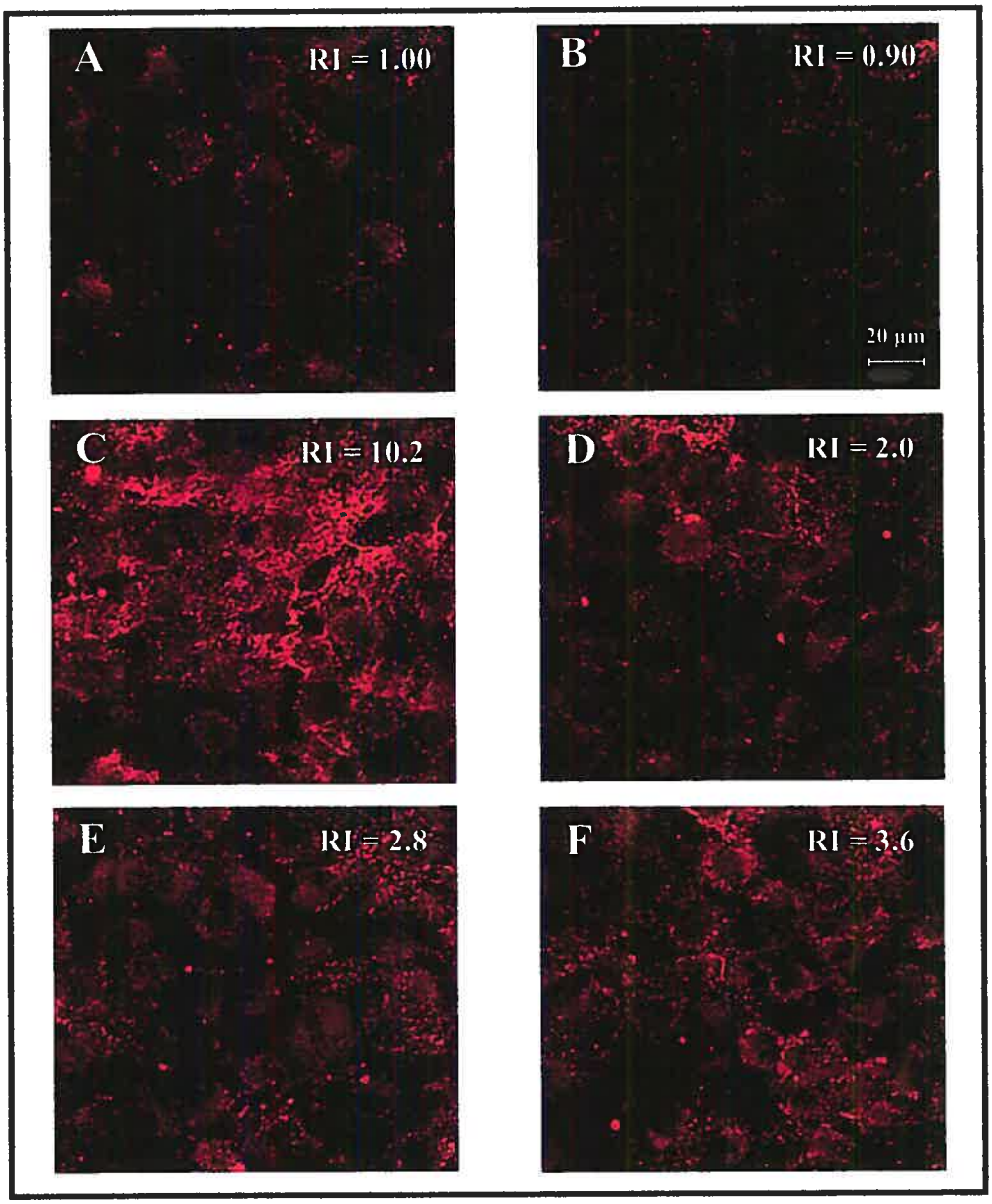
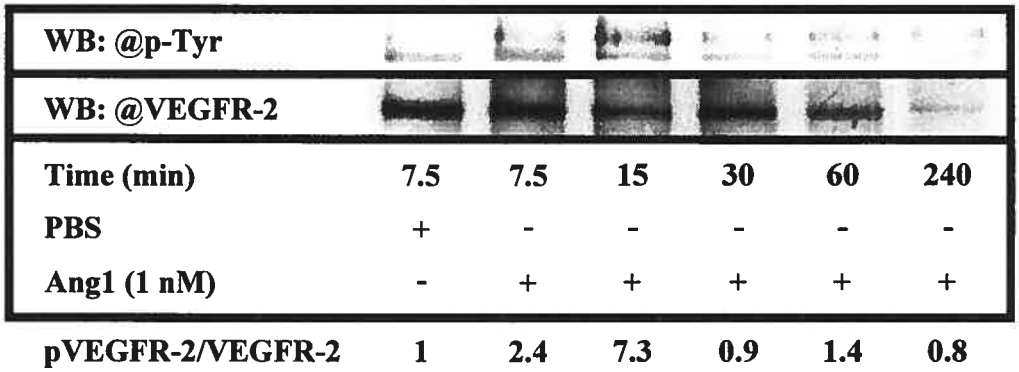


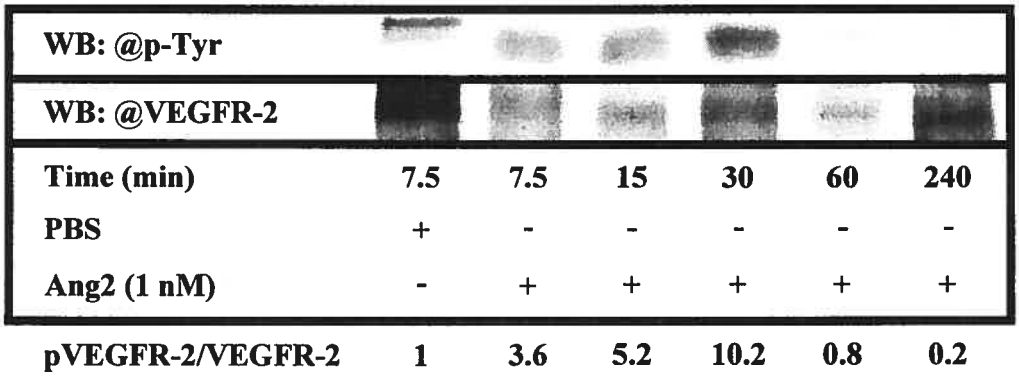
Figure 7

(A) IP: @VEGFR-2 200 kDa



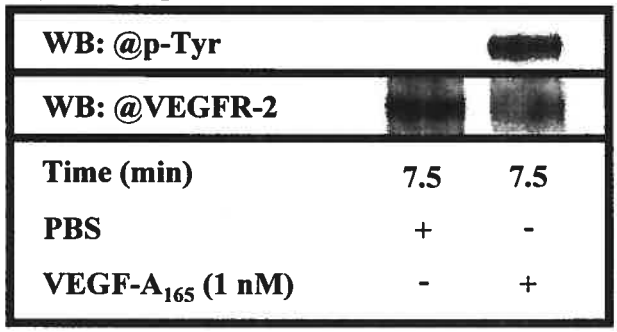
WB: @p-Tyr						
WB: @VEGFR-2						
Time (min)	7.5	7.5	15	30	60	240
PBS	+	-	-	-	-	-
Ang1 (1 nM)	-	+	+	+	+	+
pVEGFR-2/VEGFR-2	1	2.4	7.3	0.9	1.4	0.8

(B) IP: @VEGFR-2 200 kDa



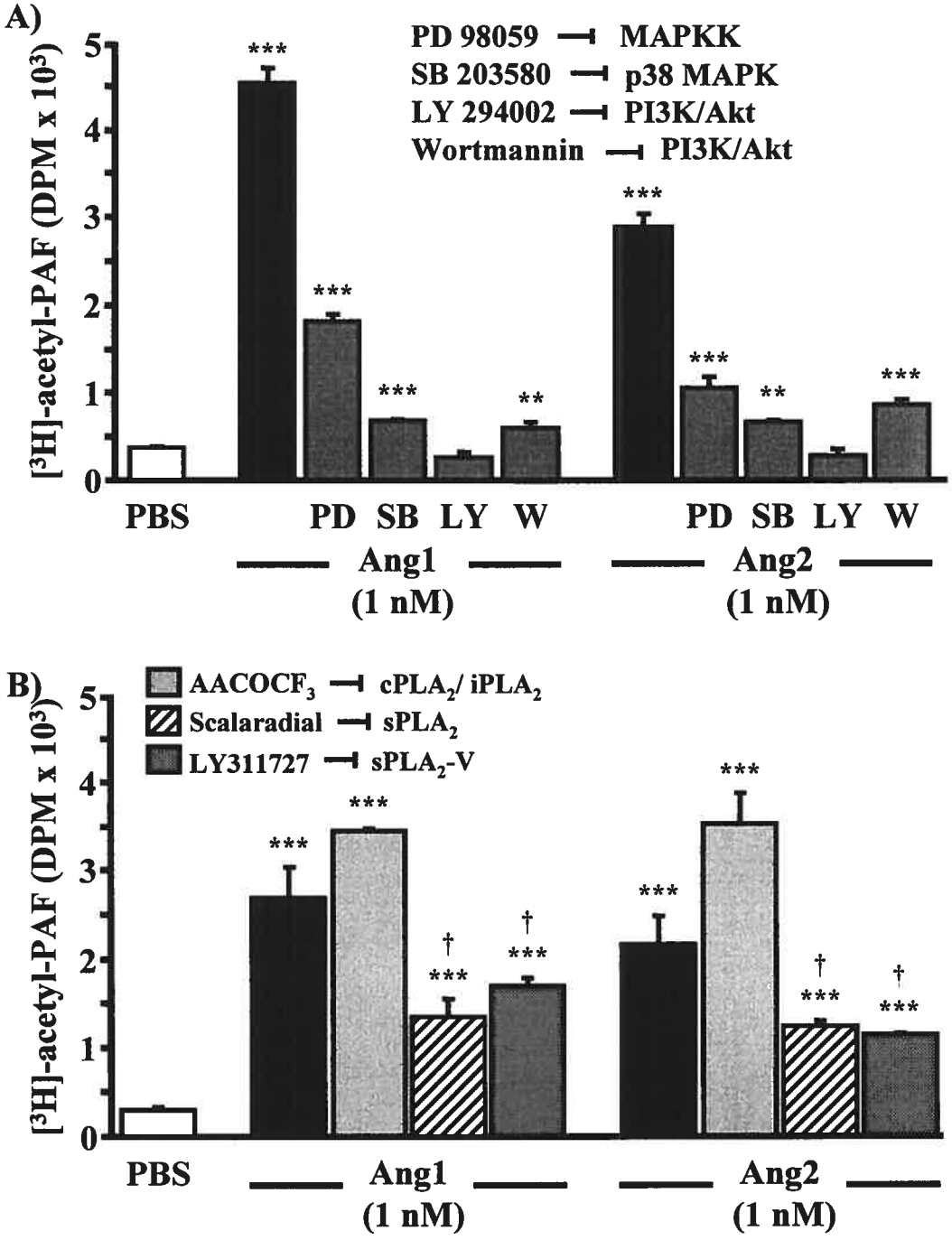
WB: @p-Tyr						
WB: @VEGFR-2						
Time (min)	7.5	7.5	15	30	60	240
PBS	+	-	-	-	-	-
Ang2 (1 nM)	-	+	+	+	+	+
pVEGFR-2/VEGFR-2	1	3.6	5.2	10.2	0.8	0.2

(C) IP: @VEGFR-2 200 kDa



WB: @p-Tyr		
WB: @VEGFR-2		
Time (min)	7.5	7.5
PBS	+	-
VEGF-A_{165} (1 nM)	-	+

Figure 8


A)
[3H]-acetyl-PAF (DPM x 10³)
PD 98059 ⊣ MAPKK
SB 203580 ⊣ p38 MAPK
LY 294002 ⊣ PI3K/Akt
Wortmannin ⊣ PI3K/Akt
PBS
PD SB LY W
PD SB LY W
Ang1 (1 nM)
Ang2 (1 nM)
B)
AACOCF3 ⊣ cPLA2/ iPLA2
Scalaradial ⊣ sPLA2
LY311727 ⊣ sPLA2-V
PBS
Ang1 (1 nM)
Ang2 (1 nM)

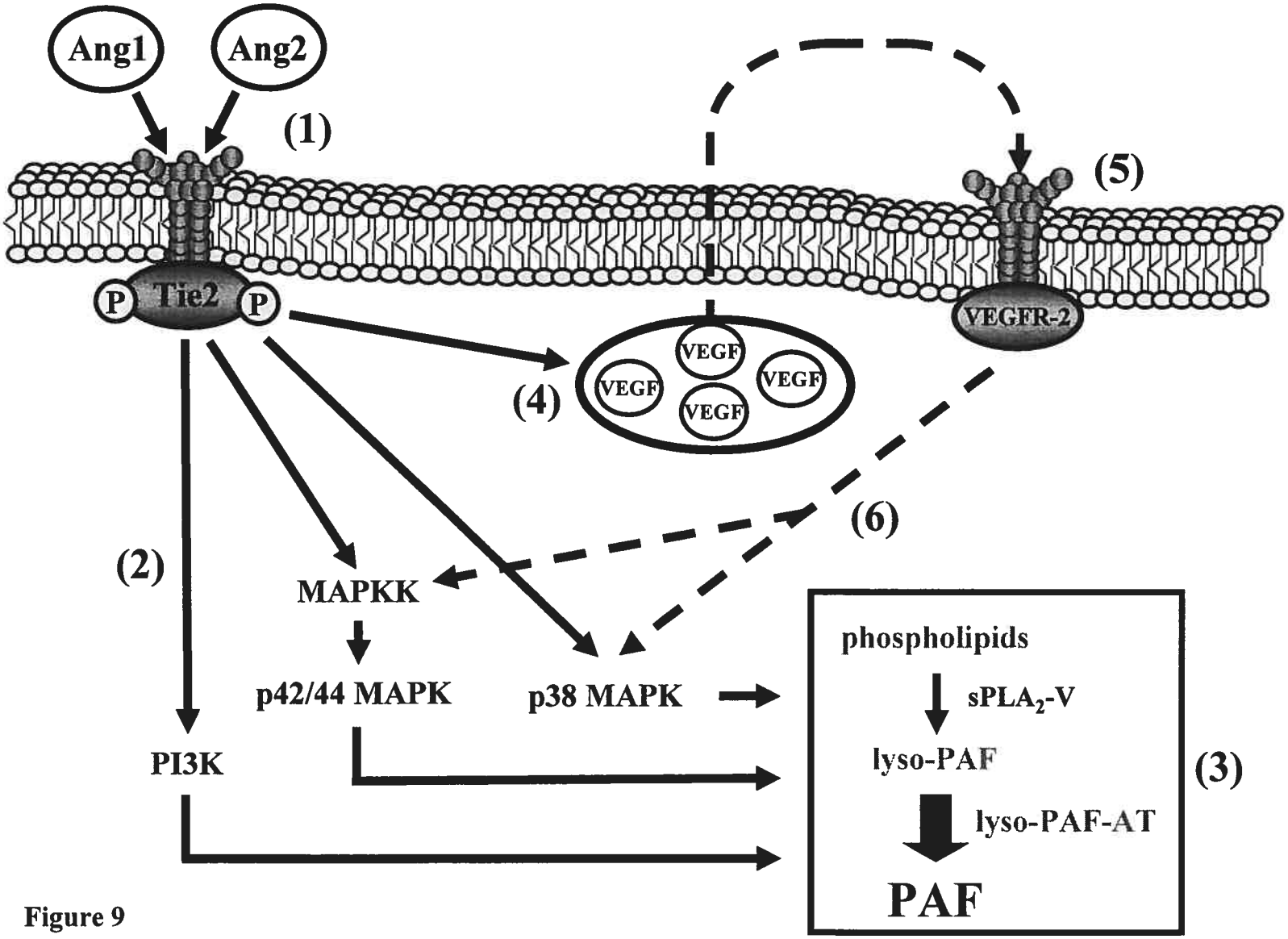

Ang1
Ang2
(1)
(5)
P
Tie2
P
VEGFR-2
VEGF
VEGF
VEGF
VEGF
(4)
(6)
(2)
MAPKK
p42/44 MAPK
p38 MAPK
PI3K
phospholipids
sPLA2-V
lyso-PAF
lyso-PAF-AT
PAF
(3)

Figure 9

3.0 Article #2

Ms. Ref. No.: CLS-D-06-00097
Title: Angiopoietin-mediated endothelial P-selectin translocation is calcium-dependent
Cellular Signalling

Dear Dr Sirois,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript.

Yours sincerely,

Susan Pyne, PhD
Co-Editor
Cellular Signalling

Reviewers' comments:

This study details evidence of the angiopoietin-induced translocation of P-selectin to the surface of BAEC cells. Both Ang1 and Ang2 are able to produce this effect which is shown to be dependent upon phospholipase C and Ca2+/calmodulin whereas the p42/p44 MAPK, p38 MAPK and PI3K pathways do not appear to be involved. However, the authors need to validate that PLCgamma is activated (rather than relying exclusively on the inhibitory effect of U73122) and to demonstrate that the various kinase inhibitors that have been used are indeed exerting the required effects under the conditions used.

1. Fig 1 is not convincing and should be amended. Firstly, which of the (apparently) three bands in the Tie2 blot (Fig 1A and Fig 1B) is Tie2? How has this been confirmed given that multiple immunoreactive bands migrate very similarly? Secondly, the 'phospho' blot in Fig 1B appears to show marked phosphorylation at 5 min, followed by a decline to 15 min. This trend is not reflected in the histogram (Fig 1C), even after taking into account the total Tie2 amount (Fig 1B, lower panel) which appears to increase with time.
2. Since the cell surface ELISA results are heavily reliant on the selectivity of the P-selectin antibody, the specificity of this reagent in BAEC should be confirmed by Western blotting.
3. It is not sufficient to rely exclusively on the reported selectivity and published IC50 values for the various inhibitors used. The authors need to substantiate their claim that Ang1 and Ang2 activate PLCgamma (e.g. by Western blotting for phosphor-PLCgamma) and to confirm (e.g. by Westerns) that the kinase inhibitors are indeed exerting the required effect under the conditions used. This is particularly important when making claims about the exclusion of certain pathways (p42/p44 MAPK, p38 MAPK and PI3K) from the translocation of P-selectin to the plasma membrane.

Identification des auteurs

1. **Identification**
   Ricardo Maliba
   Département de pharmacologie, Faculté de medicine

2. **Description de l'article**
   Titre: *Angiopoietin-mediated endothelial P-selectin translocation is calcium-dependent.*

   Liste des auteurs: Ricardo Maliba, Alexandre Brkovic, Paul-Eduard Neagoe, Louis R. Villeneuve, et Martin G. Sirois.

   Soumission à la revue: *Blood*

3. **Déclaration de tous les coauteurs autres que l'étudiant**

À titre de coauteur de l'article identifié ci-dessus, je suis d'accord pour que Ricardo Maliba inclus cet article dans sa **Identification des auteurs**
thèse de doctorat qui a pour titre *Regulation of the proinflammatory properties of angiopoietins.*

Alexandre Brkovic [redacted] 6 janvier 2006
Coauteur Signature Date

Paul-Eduard Neagoe [redacted] 6 janvier 2006
Coauteur Signature Date

Louis R. Villeneuve [redacted] janvier 2006
Coauteur Signature Date

Martin G. Sirois [redacted] 6 janvier 2006
Coauteur Signature Date

Angiopoietin-mediated endothelial P-selectin translocation is calcium-dependent

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Louis R. Villeneuve, and Martin G. Sirois*

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Running title: Angiopoietins induce P-selectin translocation

Category:

Word count: 4380; Abstract: 180

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3.1 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 favouring 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. Since 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 (BAEC) and dissect the mechanisms implicated. P-selectin, an adhesion molecule found in the Weibel-Palade bodies (WPB) of endothelial cells (EC), is rapidly translocated to the cell surface upon EC activation during inflammatory processes. Herein, we report that Ang1 and Ang2 (1 nM) are both capable of mediating a rapid Tie2 phosphorylation as well as a rapid and transient endothelial P-selectin translocation maximal within 7.5 minutes (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 (PLC-γ) activation.

Key words: angiopoietins, P-selectin, Tie2, calcium, and inflammation,

3.2 INTRODUCTION

Angiogenesis, the formation of new blood vessels from preexisting vasculature, is a tightly regulated process requiring the action of various growth factors, namely, vascular endothelial growth factor (VEGF) and angiopoietins ([1] for review). Angiogenesis plays a critical role in several pathological conditions, including atherosclerosis, proliferative retinopathies, and tumour 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 presence of VEGF-A_{165} [5]. 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 [4]. However, under certain circumstances, Ang2 may induce Tie2 phosphorylation and biological activities such as endothelial cell (EC) migration, neutrophil activation, vascular permeability, and in vitro tubule capillary-like formation [6-10].

Neutrophils are the first cells recruited to the site of inflammation and provide cytokines, growth factors, and proteolytic enzymes, which together, contribute to

trigger and support angiogenic activities [11, 12]. 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 various adhesion molecules located on the surface of EC and neutrophils. Stimulation of EC with inflammatory mediators such as thrombin, histamine, and VEGF-A_{165}, can promote a rapid and transient translocation of P-selectin contained in Weibel-Palade bodies (WPB) to the cell surface [13, 14]. P-selectin is then able to interact with its high affinity counterreceptor, P-selectin-glycoprotein-ligand-1 (PSGL-1) expressed on neutrophils and promote their rolling and transient adhesion [13-15]. Inflammatory mediators may also lead to an equivalent rapid and transient synthesis of platelet-activating factor (PAF) by EC and/or neutrophils. Newly synthesized PAF can then bind to its receptor expressed on neutrophils, and induce a rapid functional upregulation of the β_2-integrin complex (CD11/CD18) favouring the binding to its endothelial counterreceptor, intracellular adhesion molecules -1 and -2 (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 [13, 16, 17] ([18] for review).

We have recently reported that VEGF-A_{165} inflammatory effects are mediated through the synthesis of platelet activating factor by endothelial cells [19] and that PAF contributes to the induction of endothelial P-selectin translocation and neutrophil adhesion onto activated EC [15]. Furthermore, our laboratory demonstrated that Ang1 and Ang2 are also both capable of promoting endothelial P-selectin translocation and neutrophil adhesion onto EC [9]. Since angiopoietins act in

concert with VEGF to modulate vascular plasticity during postnatal neovascularization [20], 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.

3.3 MATERIAL & 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

Endothelial cells were isolated from fresh bovine aortas (BAEC) and seeded in flat-bottom 96-well plates (Fisher Scientific, Nepean, ON, Canada) and cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Burlington, ON, Canada) containing 5% fetal bovine serum (FBS; Medicorp Inc., Montreal, QC, Canada) and 1% antibiotics (Penicillin and Streptomycin; Sigma). BAEC were characterized as previously described [19, 21] and used between passages 2 and 5.

Western blot analysis of Tie2 phosphorylation

Confluent BAEC in 100 mm culture plates were serum-starved in DMEM with antibiotics overnight, rinsed with Hank's balanced salt solution (HBSS; Life Technologies), placed on ice for 30 minutes, and then stimulated in a solution of HBSS/HEPES (10 mM, pH 7.4), bovine serum albumin (BSA; 1 mg/mL; Sigma), and $CaCl_2$ (1 mM) containing Ang1 or Ang2 (1 nM) at 37°C. Cells were solubilized with lysis buffer, scraped, and the protein concentration determined by 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 anti-phosphotyrosine 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). The density of the bands was determined using Quantity One software (Bio-Rad, Mississauga, ON, Canada).

Cell surface ELISA

Endothelial P-selectin translocation was measured by cell surface enzyme-linked immunosorbent assay (ELISA) as described previously [9, 15], following stimulation of BAEC with angiopoietin -1 or -2. Briefly, BAEC were seeded and grown up to 1 day post-confluence 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 minutes prior to stimulation with angiopoietins (R&D Systems, Minneapolis, MN, USA) or VEGF-A_{165} (PeproTech Inc., Rocky Hill, NJ, USA). Reactions were stopped by removing

stimulation medium and adding 1% paraformaldehyde for 20 minutes. Following a rinse with DPBS, cells were incubated with blocking solution (5% BSA in DPBS) for 15 minutes. Cells were then incubated with rabbit polyclonal anti-human P-selectin IgG (Research Diagnostics Inc; Flanders, NJ, USA; 1:1000 dilution) for 90 minutes, rinsed with DPBS, and then incubated with HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnologies; 1:5000 dilution) for 45 minutes. 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). Due to slight variations of basal P-selectin translocation between experiments, data were reported as relative P-selectin translocation [9, 15].

Confocal Microscopy

Immunoflorescence: BAEC were grown on glass coverslips, rinsed with DPBS (37°C), stimulated with VEGF-A_{165}, Ang1, Ang2 (1 nM) or phorbol myristate acetate (PMA; 1 μM) and fixed with 1% paraformaldehyde-DPBS solution. Following fixation, the cells were incubated for 60 minutes in the dark with wheat germ agglutinin (WGA) conjugated to Alexa 488 (1:200 dilution; Invitrogen) 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 minutes. 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 minutes. Cells were rinsed with DPBS and incubated with donkey anti-rabbit

conjugated to Alexa 555 IgG (1:600 dilution; Invitrogen) for 60 minutes. Glass coverslips were mounted using 1,4-diazabicyclo-2-2-2-octane (DABCO)/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 63x/1.4 Plan-Apochromat objective (Zeiss) was used for magnification. Voxel size is 71 nm X 71 nm X 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 stacks 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 (QCT) between iterations. Deconvolved Z stacks were saved in Tiff files format series or ICS files and transferred to the LSM 510 or SFP (Simulated Fluorescence Process, SVI) software, respectively. Front view transparent projections were made using 4 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. These projections 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 Analysis

Data are mean ± SEM. Comparisons were made by analysis of variance followed by a Bonferroni t-test using GraphPad Instat version 3.05 (GraphPad Software Inc.). Data were considered significantly different if values of $p<0.05$ were observed.

3.4 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 minutes, corresponding to a 2.2-fold increase upon 15 minutes of treatment *versus* control DPBS-treated cells (Figure 1A). Similarly, treatment with Ang2 (1 nM) also induced within 7.5 minutes Tie2 phosphorylation and corresponding to a 4.1-fold increase following a 15 minute treatment compared to control DPBS-treated cells (Figure 1B).

Ang1 and Ang2 induce P-selectin translocation in BAEC

We have previously reported that VEGF-A_{165} requires endothelial PAF synthesis to promote P-selectin translocation in human umbilical vein endothelial cells (HUVEC) [15]. In addition, we observed that both Ang1 and Ang2 are capable of inducing P-selectin translocation in HUVEC, and this, in absence of endothelial PAF synthesis [9]. More recently, we demonstrated that Ang1 and Ang2 are capable of mediating PAF synthesis in BAEC [22] 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 minutes) and concentration-dependent manner (1 pM – 5 nM). Peak values (125% increase) were observed within 7.5 minutes and at 1 nM as compared to control DPBS-treated cells (Figure 2A and B). Similarly, treatment of BAEC with

Ang2 (1 nM) induced a 100% increase in endothelial P-selectin translocation within 7.5 minutes at (Figure 2C and D). As positive control, BAEC were stimulated with VEGF-A_{165} (1 nM) for 7.5 minutes increasing by 200% basal endothelial P-selectin translocation (Figure 2).

To confirm the ability of angiopoietins to promote endothelial P-selectin translocation, we used a confocal microscopy approach. Translocation of P-selectin to the non-permeabilized endothelial cell 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. 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 (Figure 3A1-A3). In control DPBS-treated BAEC, we observed a basal level of P-selectin at the cell surface membrane (Figure 3B1-B3). Treatment with positive controls (PMA; 1 μM and VEGF-A_{165}; 1 nM), or with Ang1 or Ang2 (1 nM), for 7.5 minutes induced P-selectin translocation along the cytoplasmic membrane (Figure 3C1-C3 to 3F1-3F3, respectively).

Non-permeabilized 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 (Figure 3A1-3F1), and transverse (Figure 3A2-3F2) 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 extracellular P-selectin translocated domain, confocal images were cumulated to

provide a three (3)-dimensional projection of the cells in which the extracellular P-selectin domain appears in red (Figure 3A3-3F3).

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 [23]. We thus sought to determine the role played by PLC-γ in angiopoietin-mediated endothelial P-selectin translocation by pretreating BAEC for 15 minutes 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 minutes). Angiopoietin-mediated endothelial P-selectin requires the activation of PLC-γ since pretreatment with U73122 reduced Ang1- and Ang2-mediated P-selectin translocation by 56% and 88%, respectively (Figure 4). Similarly, inhibition of PKC, activated immediately downstream of PLC-γ, also reduced endothelial P-selectin translocation in BAEC treated with Ang1 and Ang2 both by 62% (Figure 4). As positive control, BAEC were pretreated with U73122 and Calphostin C prior to stimulation with VEGF-A_{165} (1 nM; 7.5 minutes). U73122 and Calphostin C diminished VEGF-A_{165}-mediated endothelial P-selectin translocation by 78% and 74%, respectively (Figure 4).

Angiopoietins mediate endothelial P-selectin translocation independently of p38 MAPK, p42/44 MAPK, PI3K activation, and PAF synthesis.

Activation of PKC may lead to the activation of p42/44 mitogen-activated protein kinase (MAPK). Furthermore, we and others have reported that Tie2 receptor

autophospho-rylation following stimulation with Ang1 or Ang2 can in turn activate p38 MAPK, p42/44 MAPK, and PI3K/Akt intracellular signaling pathways [6, 22, 24-28]. Pretreatment of BAEC with selective inhibitors for MAPKK (PD98059; 10 μM), p38 MAPK (SB203580; 10 μM), or PI3K (Wortmannin; 500 nM) prior to stimulation with angiopoietins did not significantly reduce P-selectin translocation (Figure 5A). However, pretreatment with the aforementioned inhibitors reduced P-selectin translocation following 7.5 minutes of stimulation with VEGF-A_{165} by 55 to 57% (Figure 5A).

In addition, we have previously reported on the role of endothelial PAF in VEGF-A_{165}-mediated P-selectin translocation in HUVEC [15]. To assess the contribution of endothelial PAF to angiopoietin-mediated P-selectin translocation, BAEC were pretreated with selective antagonists for either intracellular (LAU 8080; 100 nM), extracellular (BN 52021; 10 μM) or both intra- and extracellular (CV-3988; 10 μM) PAF receptors (PAFR) 15 minutes prior to stimulation with either Ang1 or Ang2 (1 nM) for 7.5 minutes. Pretreatment with PAFR antagonists had no effect on angiopoietin-mediated P-selectin translocation (Figure 5B). 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 (Figure 5B).

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 PI3K intracellular signalling pathways nor by PAF. However, exocytosis of WPB and the subsequent translocation of P-selectin has been shown to be calcium-dependent following stimulation of EC with VEGF-A_{165} [23]. To determine which calcium stores are mobilized during angiopoietin-mediated P-selectin translocation, BAEC were pretreated with an intra- and extracellular calcium chelator (BAPTA-AM; 10 μM) or stimulated in Ca^{2+}-free DPBS. Chelation of intra- and extracellular calcium completely abrogated endothelial P-selectin translocation. In addition, in an extracellular Ca^{2+}-free environment, we observed that the release of Ca^{2+} from intracellular pools was insufficient to support endothelial P-selectin translocation (Figure 6A). No significant statistical differences were observed between BAPTA-AM-treated and Ca^{2+}-free DPBS groups for each of the three growth factors studied.

In order to determine the role of the calcium/calmodulin complex (Ca^{2+}/CaM) on angiopoietin-mediated endothelial P-selectin translocation, we disrupted 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 (Figure 6B). Similarly, VEGF-A_{165}-mediated endothelial P-selectin translocation was also completely inhibited in response to pretreatment with W-7 (Figure 6B).

3.5 DISCUSSION

In the present study, we demonstrate that Ang1 and Ang2 are both 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 calcium-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 Tie2 receptor [3, 4, 7, 25], whereas Ang2 was described as a natural Tie2 antagonist in EC [4]. However, other studies reported that the use of high concentrations or prolonged treatment with Ang2 can induce Tie2 activation, support endothelial cell survival and EC tubule capillary-like formation [6, 7]. More recently, we and others reported that Ang2 can also promote a rapid Tie2 phosphorylation (within minutes) [8, 9, 22], mediate tubule capillary-like formation in immortomouse brain endothelial cells [8], endothelial P-selectin translocation, and neutrophil adhesion onto activated HUVEC [9]. 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 from

Weibel-Palade bodies (WPB) upon stimulation with various inflammatory mediators as rapidly as 2 minutes after stimulation with a peak within 10 minutes [29, 30] whereupon P-selectin is removed from the cell surface either by enzymatic cleavage or by endocytosis to be recycled back into WPB [31]. In addition, we demonstrate that endothelial P-selectin translocation mediated by angiopoietins is concentration-dependent. Indeed, maximal P-selectin translocation was attained at 1 nM, and interestingly, at a higher concentration (5 nM), the capacity of Ang1 and Ang2 to mediate P-selectin translocation was reduced. 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 (PGI_2) synthesis in BAEC [9, 15, 22, 32]. 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 [33]. However, an overabundance of ligand impedes receptor dimerization [34]. 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 and other tyrosine kinase receptor ligands.

Intracellular signalling implicated in angiopoietin-mediated P-selectin translocation. In previous studies, we defined that the inflammatory properties of VEGF-A_{165} requires endothelial PAF synthesis. In addition, we reported that VEGF-A_{165}-mediated PAF synthesis implies the dual activation of PLC-γ/PKC/p42/44 MAPK and MLK/MKK-3, 6/p38 MAPK signalling pathways [35]. We also reported that VEGF-A_{165} induces P-selectin translocation through endogenous PAF synthesis in HUVEC [15]. Furthermore, we recently observed that both Ang1 and Ang2 can promote P-selectin translocation in absence of PAF synthesis in HUVEC [9]. However, we recently observed that Ang1 and Ang2 as VEGF-A_{165} can mediate PAF synthesis in BAEC [22]. Finally, recent findings indicate that VEGF-A_{165}-mediated WPB exocytosis is regulated in part through PLC-γ signal transduction [23]. We therefore sought to dissect the signalling pathways implicated in angiopoietin-mediated P-selectin translocation and the role of endogenous PAF in BAEC using VEGF-$_{A165}$ as a positive control.

In the current study, we demonstrate that angiopoietin-mediated endothelial P-selectin translocation requires PLC and PKC activation. Indeed, treatment of BAEC with selective inhibitors of PLC-γ (U73122) or PKC (Calphostin C) prior to stimulation with Ang1 or Ang2 reduced P-selectin translocation. Similarly, VEGF-A_{165}-mediated P-selectin translocation is also dependent on the activation of PLC-γ and PKC. PLC-γ is an important regulator of calcium signalling and PKC, immediately downstream, is implicated in p42/44 MAPK activation which in turn regulates endothelial PAF synthesis. Taken together, these observations prompted us

to investigate calcium signalling and intracellular signalling pathways leading to endothelial PAF synthesis.

Since Ang1 and Ang2, upon binding to Tie2, have been shown to activate p42/44 MAPK, p38 MAPK, and PI3K intracellular signalling pathways [6, 22, 24-28] leading to a rapid and sustained synthesis of PAF in BAEC [22], we wished to determine whether these pathways and PAF were implicated in angiopoietin-mediated P-selectin translocation. In the current study, we demonstrate that the aforementioned pathways are not implicated in angiopoietin-mediated endothelial P-selectin translocation and neither is endothelial PAF. On the other hand, VEGF-A_{165}-mediated translocation requires the activation of p38 MAPK and p42/44 MAPK, both capable of mediating endothelial PAF synthesis and PI3K which, although activated, has been shown not to increase PAF synthesis [35]. When we pretreated BAEC with selective PAFR antagonists, we observed a reduction in translocation, although not as pronounced as what we had previously reported in HUVEC [15]. Taken together, our data demonstrates that the 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.

The translocation of P-selectin and constituents of WPB requires the movement of the WPB from the cytoplasm to the cell membrane and the fusion of these vesicles

with the plasma membrane. Increased levels of cytosolic free Ca^{+2} concentration ($[Ca^{2+}]_i$) levels have been implicated in the mechanism of exocytosis for a number of agonists including thrombin and histamine [36]. The cellular responses to increased $[Ca^{2+}]_i$ are most likely mediated through calmodulin and small GTP-binding proteins [37-39]. In our current study, treatment of BAEC with an intracellular calcium chelator (BAPTA-AM) or a calmodulin inhibitor (W-7) prior to stimulation with Ang1 or Ang2 completely abrogated P-selectin translocation. Furthermore, P-selectin translocation was absent in cells treated with Ang1 or Ang2 in calcium-free DPBS. Together, these data confirm the importance of Ca^{2+} in P-selectin translocation mediated by angiopoietins. It has been established that elevation of Ca^{2+} over basal levels (100 nM) is required for regulated exocytosis [40, 41] and that this elevation may be due to influx of calcium across the plasma membrane [42], from internal stores, or both [43, 44]. The endoplasmic reticulum (ER) is the best characterized Ca^{2+} store in mammalian cells [45] and release of Ca^{2+} from the ER can be triggered by activation of inositol triphosphate (IP_3) [46] which can also modulate calcium release from the Golgi complex [47]. In our current study we show that PLC-γ, which can act on PIP_2 to produce IP_3 and hence trigger Ca^{2+} release from intracellular stores, regulates angiopoietin-mediated endothelial P-selectin. Once released, calcium may form a complex with calmodulin. This complex has been shown to interact with a small GTP-binding protein, Ral, in a calcium-dependent manner and play an important role in regulating WPB exocytosis in EC [36]. In fact, we observed that chelation of intra- and extracellular calcium completely abrogated endothelial P-selectin translocation. Furthermore, we observed that the release of

Ca^{2+} from intracellular pools was insufficient to support endothelial P-selectin translocation in an extracellular Ca^{2+}-free environment. Taken together, these observations demonstrate the critical role of calcium in angiopoietin-mediated endothelial P-selectin translocation. Using another approach, namely inhibiting the formation of the Ca^{2+}/calmodulin complex with W-7 and this, regardless of intra- or extracellular levels of calcium, reduces translocation to levels below control values confirming the importance of calcium in P-selectin translocation. Similarly, calcium also contributes to mediate VEGF-A_{165}-induced endothelial P-selectin translocation.

Recent studies have reported that Ang1 possesses anti-inflammatory properties in vitro, namely by reducing VEGF-induced leukocyte adhesion onto HUVEC and E-selectin expression [48] and thrombin-mediated neutrophil adhesion onto EC [49]. Upon initial review, these results may appear contradictory to what we report herein as well as what we have previously demonstrated [9] but both of the above studies [48, 49] were performed for extended periods of time that are well in excess of the timeframe of P-selectin activity. Until recently, the role of Ang2 in inflammation was unknown. In addition to our recent demonstration that Ang2 (as well as Ang1) promotes neutrophil PAF synthesis, endothelial P-selectin translocation, and neutrophil adhesion onto EC [9], Ang2 has been shown to promote vascular leakage in vivo [10]. Furthermore, Ang2 has been shown to be stored exclusive of P-selectin and rapidly released from WPB upon stimulation with PMA and calcium ionophores but not with VEGF or Ang1 [50]. Taken together, these observations demonstrate that the angiopoietins should be considered as acute proinflammatory mediators.

In summary, we demonstrate that in BAEC, Ang1 and Ang2 induce a rapid and transient translocation of P-selectin dependent on calcium entry and PLC-γ activation. However, contrary to VEGF-A_{165}-mediated P-selectin translocation, angiopoietins do not require the synthesis of endogenous PAF. P-selectin translocation plays a preponderant role in neutrophil rolling, an important event in acute inflammation. This study allowed us to gain a better understanding of the intracellular mechanisms mediating angiopoietin-induced endothelial P-selectin translocation.

3.6 ACKNOWLEDGEMENTS

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3.8 FIGURE LEGENDS

Figure 1: Ang1 and Ang2 activate Tie2 in a time-dependent manner. Confluent BAEC were treated with Ang1 (A), or Ang2 (B) from 5 to 15 minutes. Cell lysates (500 μg) were prepared and immunoprecipitated (IP) 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 (WB) analysis following incubation with rabbit polyclonal anti-mouse Tie2 IgG. Relative Tie2 phosphorylation over Tie2 total protein expression under various experimental conditions was calculated by normalizing DPBS control optical density (OD) values to 1 (C).

Figure 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 enzyme-linked immunosorbent assay (ELISA). Solution buffer (DPBS) was used as control and the basal levels of P-selectin translocation were normalized to 1. Maximal P-selectin translocation was observed at 7.5 minutes (A) at a concentration of 1 nM (B). Similarly, Ang2-mediated endothelial P-selectin translocation was maximal at 7.5 minutes (C) and at 1 nM (D). As positive control, BAEC were stimulated with VEGF-A_{165} (1 nM; 7.5 minutes). Data are expressed as relative absorbance measured

at 450 nm. Values are means ± SEM of at least 3 experiments. ** and *** denote $p < 0.01$ and 0.001 vs. DPBS, respectively.

Figure 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 minutes. 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. Presence of P-selectin within the cell surface membrane appears in yellow. Front view transparent projections (A1-F1) were made using 4 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 (3)-dimensional representation of the cells (A3-F3) was performed by using the Simulated Fluorescence Process (SFP) software, projections were created using all the slices of the Z stacks. Magnification = 63x; digital zoom = 2x, Bar = 5 μm.

Figure 4: Endothelial P-selectin translocation mediated by Ang1 or Ang2 requires PLC-γ and PKC signal transduction. Confluent BAEC were pretreated with selective pharmacological inhibitors for phospholipase C-γ (PLC-γ; U73122; 10 μM) and protein kinase C (PKC; Calphostin C; 100 nM) for 15 minutes prior to 7.5 minutes of stimulation with Ang1 or Ang2 (1 nM). BAEC were also stimulated with

VEGF-A_{165} (1 nM) for 7.5 minutes as 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 at least 3 experiments. ** and *** denote $p < 0.01$ and $p < 0.001$ vs. PBS, respectively; †, ††, and ††† represent $p< 0.05$, $p< 0.01$, and $p< 0.001$ vs. agonist, respectively.

Figure 5: p38 MAPK, p42/44, and PI3K signalling 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 PI3K (Wortmannin; 500 nM) for 15 minutes prior to 7.5 minutes of stimulation with Ang1 or Ang2 (1 nM). (B) In a separate series of experiments, confluent BAEC were pretreated with antagonists targeting either the intracellular (LAU 8080; 100 nM), extracellular (BN 52021; 10 μM) or both intra- and extracellular (CV-3988; 10 μM) PAF receptors (PAFR) for 15 minutes prior to stimulation with either Ang1 or Ang2 (1 nM) for 7.5 minutes. BAEC were stimulated with VEGF-A_{165} (1 nM) for 7.5 minutes as positive control. Values are means ± SEM of at least 3 experiments. ** and *** denote $p < 0.01$ and $p < 0.001$ vs. PBS, respectively; †† and ††† represent $p< 0.01$ and $p< 0.001$ vs. agonist, respectively.

Figure 6: Angiopoietin-mediated endothelial P-selectin requires calcium. Confluent BAEC were pretreated with an intracellular calcium 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 inhibitor (W-7; 10 mM) prior to treatment with Ang1 or Ang2 (1 nM). VEGF-A_{165} (1 nM; 7.5 minutes) is present as 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 at least 3 experiments. * and *** denote $p < 0.05$ and $p < 0.001$ vs. PBS, respectively; †, ††, and ††† represent $p< 0.05$, $p< 0.01$, and $p< 0.001$ vs. agonist, respectively.

3.9 FIGURES

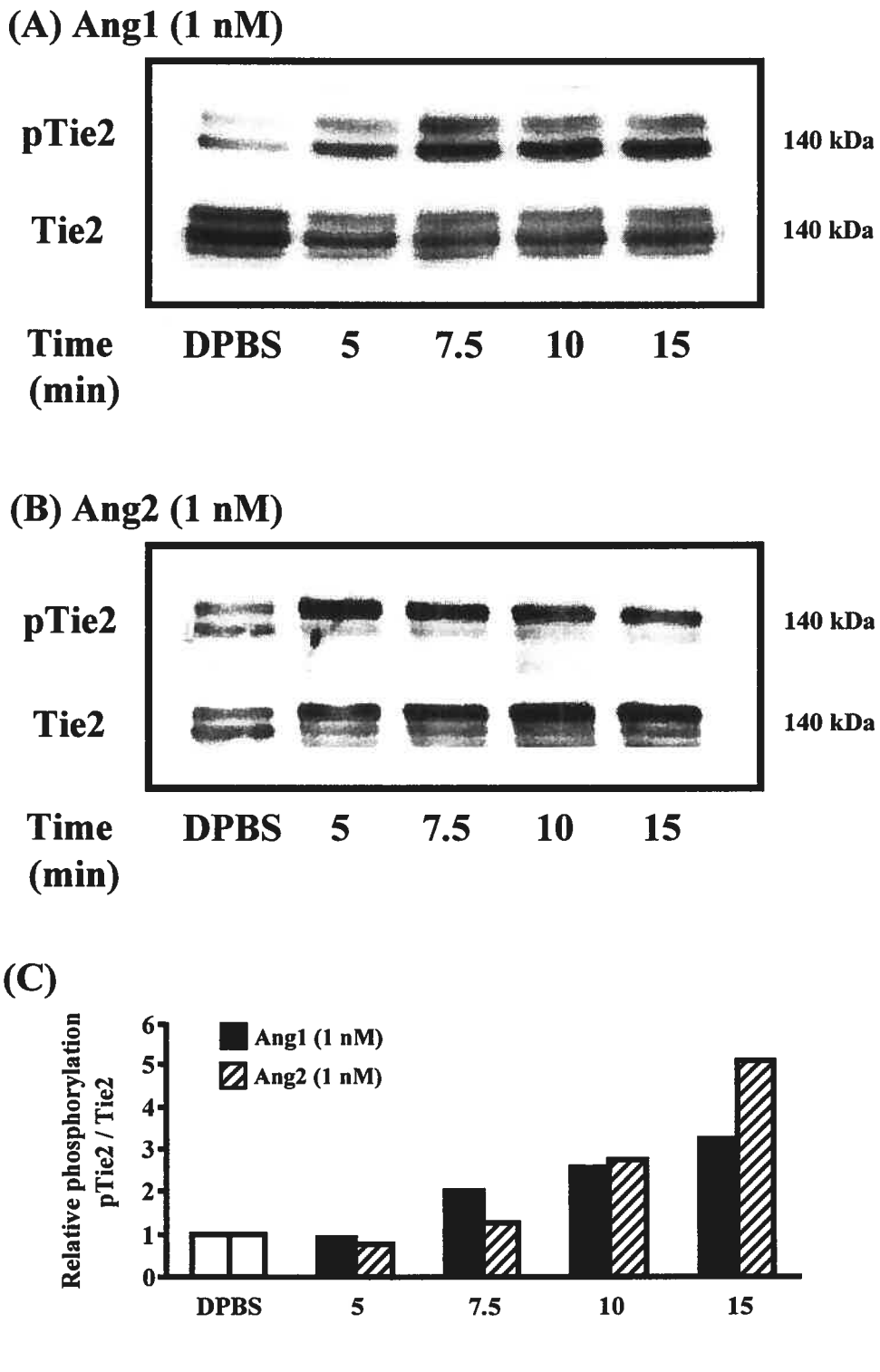

Figure 1

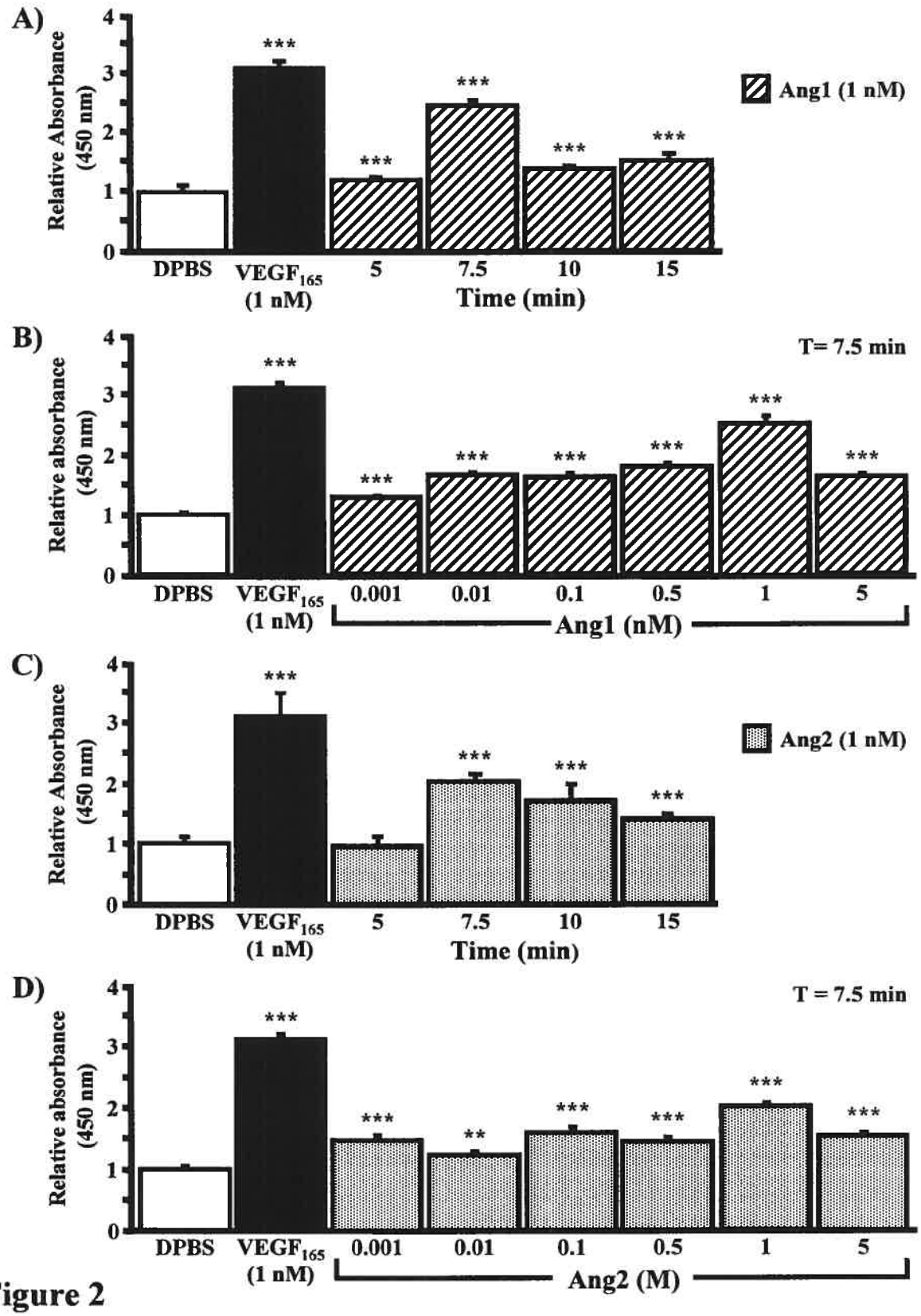
A)
Relative Absorbance (450 nm)
Ang1 (1 nM)
DPBS
VEGF165 (1 nM)
5
7.5
10
15
Time (min)
B)
Relative absorbance (450 nm)
T= 7.5 min
0.001
0.01
0.1
0.5
1
5
Ang1 (nM)
C)
Relative Absorbance (450 nm)
Ang2 (1 nM)
Time (min)
D)
Relative absorbance (450 nm)
T = 7.5 min
Ang2 (M)

Figure 2

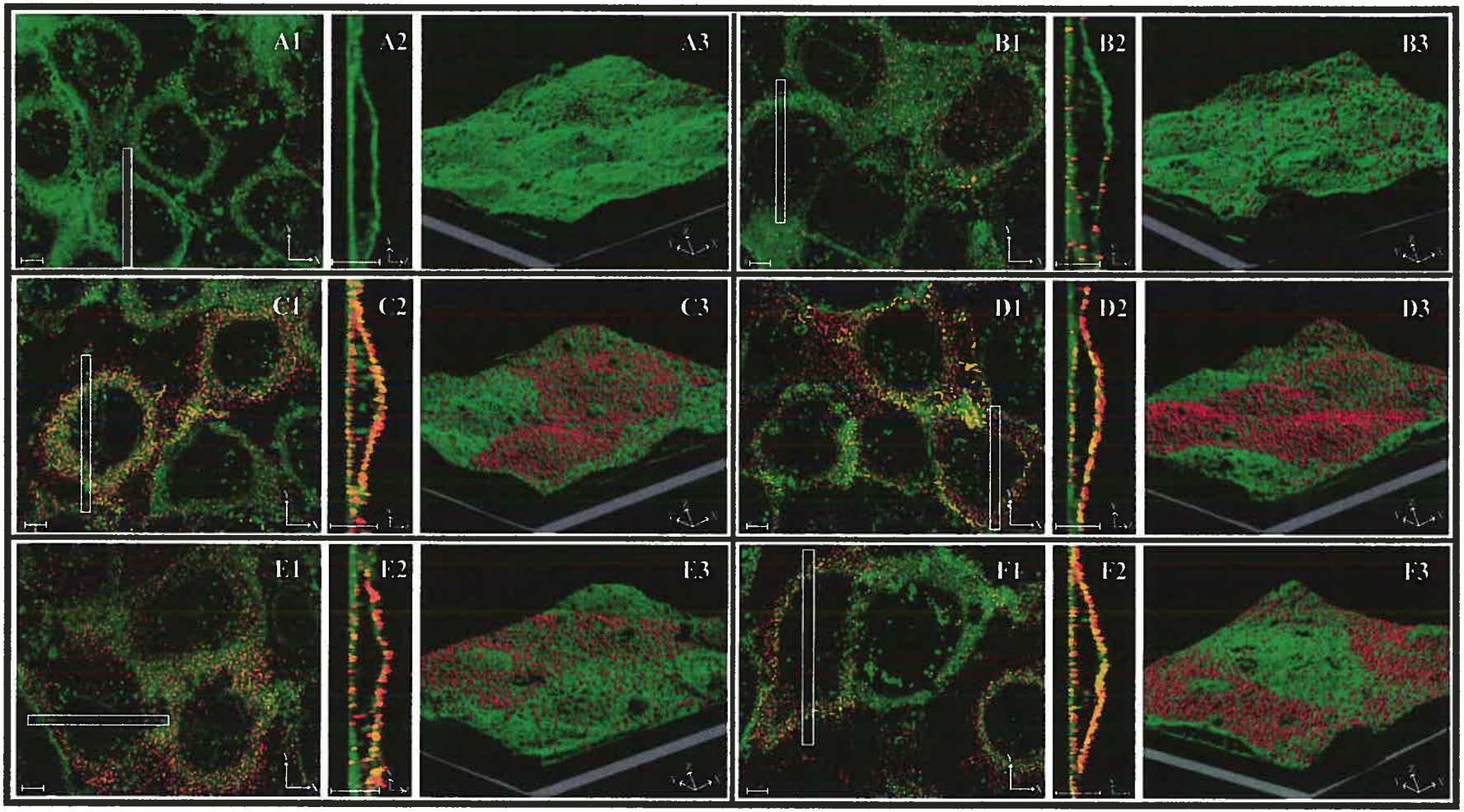

Figure 3

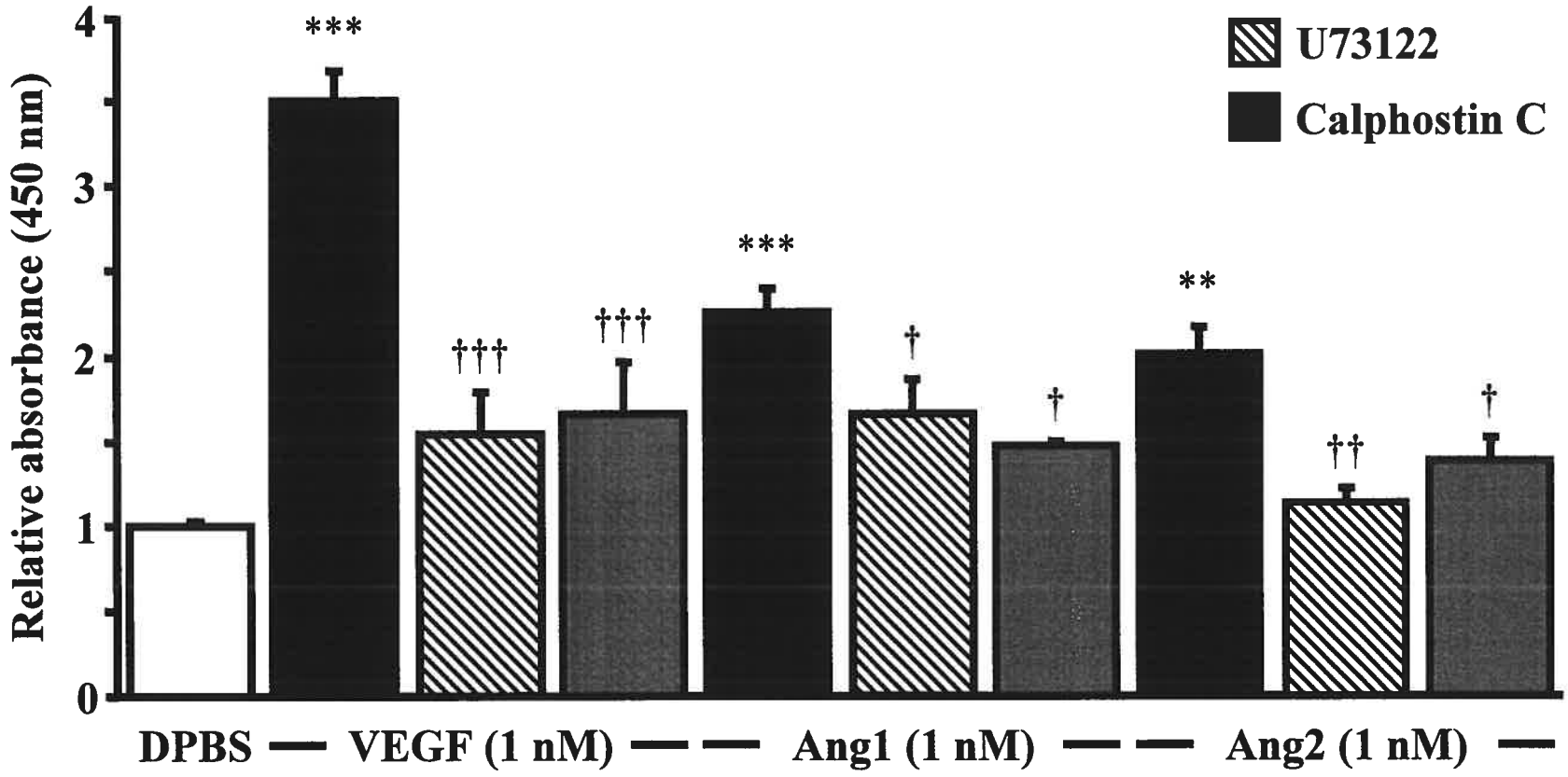
Relative absorbance (450 nm)
4
3
2
1
0
***
***
**
†††
†††
†
†
†
††
†
U73122
Calphostin C
DPBS
VEGF (1 nM)
Ang1 (1 nM)
Ang2 (1 nM)

Figure 4

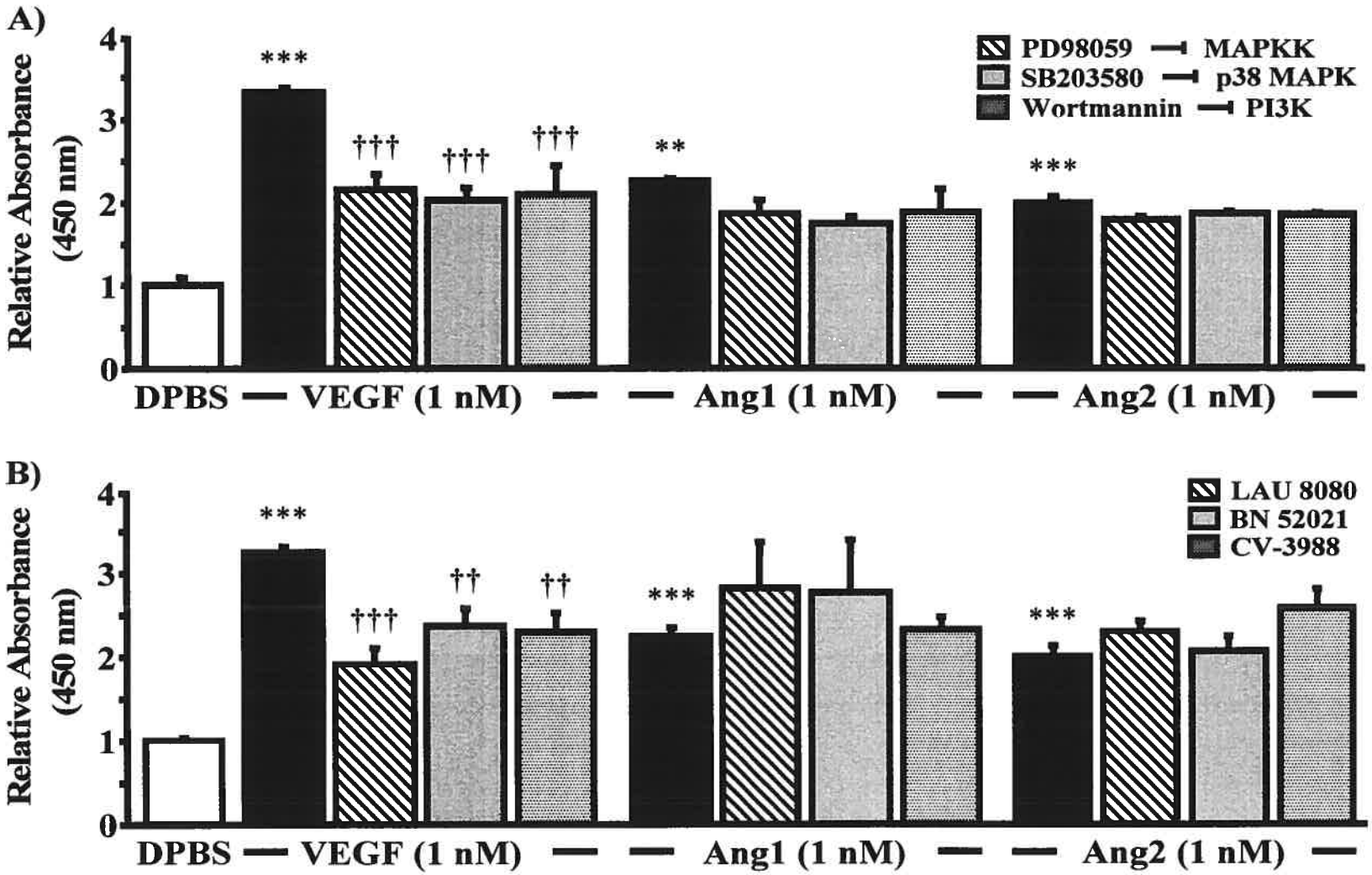
A)
Relative Absorbance (450 nm)
PD98059 — MAPKK
SB203580 — p38 MAPK
Wortmannin — PI3K
***
†††
†††
†††
**
***
DPBS
VEGF (1 nM)
Ang1 (1 nM)
Ang2 (1 nM)
B)
Relative Absorbance (450 nm)
LAU 8080
BN 52021
CV-3988
***
†††
††
††
***
***
DPBS
VEGF (1 nM)
Ang1 (1 nM)
Ang2 (1 nM)

Figure 5

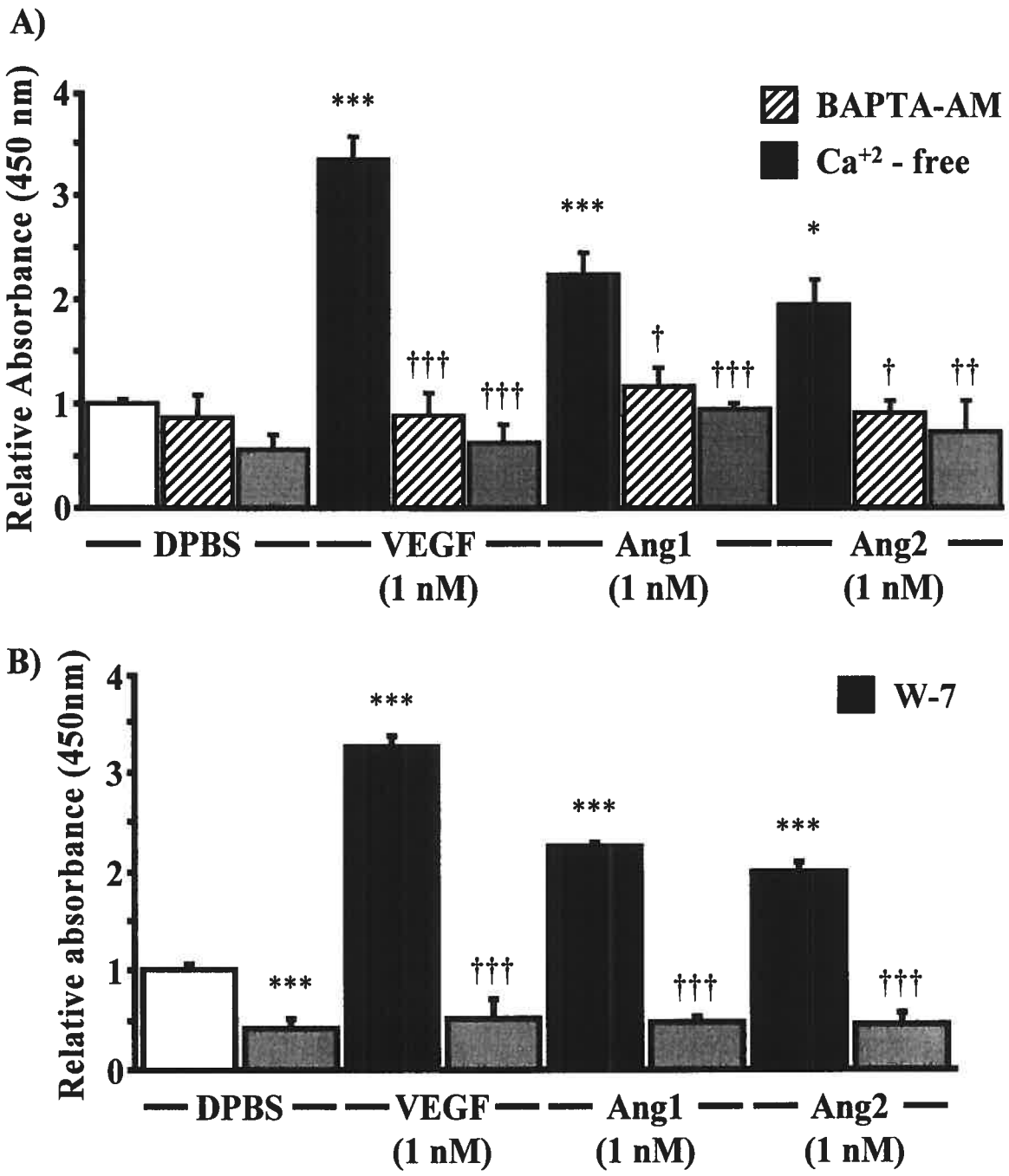
A)
Relative Absorbance (450 nm)
BAPTA-AM
Ca+2 - free
DPBS
VEGF (1 nM)
Ang1 (1 nM)
Ang2 (1 nM)
B)
Relative absorbance (450nm)
W-7
DPBS
VEGF (1 nM)
Ang1 (1 nM)
Ang2 (1 nM)

Figure 6

4.0 Discussion

Our laboratory has reported that the effect of VEGF-A_{165} on vascular permeability is mediated through the synthesis of platelet activating factor (PAF) by endothelial cells [56]. In addition, we have delineated the intracellular signalling pathways leading to VEGF-A_{165}-mediated endothelial PAF synthesis [95,230,274] in BAEC. Furthermore, we have recently demonstrated that VEGF-A_{165} induces endothelial P-selectin translocation and the subsequent adhesion of neutrophils onto activated endothelial cells through the synthesis of endogenous PAF [65] in HUVEC.

Recently identified, the angiopoietins (Ang1 and Ang2), are defined as ligands for the tyrosine kinase receptor Tie2 [24,25] to which they bind with similar specificity and affinity [24,25]. Ang1 has been characterized as a Tie2 agonist, having the capacity to stabilize and promote the maturation of unstable vessels in presence of VEGF-A_{165} [123]. 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 [25]. However, under certain circumstances, Ang2 may induce Tie2 phosphorylation and biological activities such as endothelial cell (EC) migration, neutrophil activation, vascular permeability, and *in vitro* tubule capillary-like formation [124,125,127,149,150]. We have recently reported that Ang1 and Ang2 are both capable of promoting endothelial P-selectin translocation and neutrophil adhesion onto activated HUVEC in the absence of PAF synthesis [149]. However, in neutrophils, Ang1 and Ang2 activated Tie2 receptors leading to PAF synthesis,

functional up-regulation of the β_2-integrin complex, and a subsequent increase in neutrophils adhesion [149].

Therefore, building upon the numerous studies performed in our laboratory investigating the role of VEGF-A_{165} on the regulation, synthesis, and signalling pathways associated with endothelial PAF and knowing that angiopoietins act in concert with VEGF to modulate vascular plasticity during postnatal neovascularization [275], we set out to determine whether the angiopoietins could modulate proinflammatory events, namely PAF synthesis and P-selectin translocation, in BAEC and identify the intracellular mechanisms implicated.

Herein, we report 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 minutes 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). In addition, we demonstrate that the angiopoietins are also capable of mediating a rapid and transient endothelial P-selectin translocation. Moreover, angiopoietin-mediated endothelial P-selectin translocation is calcium-dependent and regulated through phospholipase C-γ (PLC-γ) activation and contrary to VEGF-A_{165}, this translocation does not require endothelial PAF synthesis.

4.1 Ang1 and Ang2 behave as Tie2 agonists in BAEC

Early studies reported that Ang1 can induce a rapid activation of Tie2 receptor [24,25,124,164], whereas Ang2 was described as a natural Tie2 antagonist in EC [25]. However, other groups reported that the use of high concentrations or prolonged treatment with Ang2 can induce Tie2 activation, support endothelial cell survival and EC tubule capillary-like formation [124,125,127]. Our data showed that Ang1 and Ang2 are both capable of mediating a rapid and transient phosphorylation of Tie2 in endothelial cells.

When determining whether the angiopoietins induced either endothelial PAF synthesis or P-selectin translocation, we set out to identify the optimal concentration with which to conduct future experiments. Hence, we observed that endothelial PAF synthesis and P-selectin translocation mediated by Ang1 and Ang2 proceed in a dose-dependent manner. Ang1 and Ang2 at 10^{-9} M had their maximal agonistic effect on PAF synthesis, increasing it by 1695% and 851%, respectively, suggesting that Ang2 might serve as a partial Tie2 agonist with respect to PAF synthesis. At a higher concentration (10^{-8} M), Ang1- and Ang2-mediated PAF synthesis was also almost completely lost. Similarly, we demonstrate that endothelial P-selectin translocation mediated by angiopoietins was also attained at 10^{-9} M, and interestingly, at a higher concentration ($5x10^{-9}$ M), the capacity of Ang1 and Ang2 to mediate P-selectin translocation was also reduced. We have also observed this phenomenon under other experimental conditions, namely for VEGF-A_{165} and angiopoietin-mediated P-

selectin translocation in HUVEC, as well as VEGF-A_{165}-mediated prostacyclin (PGI_2) synthesis in BAEC [65,80,149]. These observations 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 [276]. However, an overabundance of ligand impedes receptor dimerization [277]. Considering that numerous studies reported the use of angiopoietins at concentrations exceeding 10^{-9} M [127,143,146,148,185] and draw conclusions that differ somewhat from ours, 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.

In this study, we also demonstrated 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 [162] as well as Akt [165]. Most studies investigating signalling downstream of Tie2 mainly focused on PI3K/Akt [143,146,164,278] 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 [279]. Interestingly, we also observed the capacity

of Ang2 to activate p38 and p42/44 MAPKs, which had yet to be documented, in addition to PI3K/Akt which had previously been described by others [127,280]. 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 [114].

4.2 Ang1 and Ang2 induce PAF synthesis in BAEC

Angiogenesis plays a critical role in several pathological conditions, namely atherosclerosis, proliferative retinopathies and tumour growth [13]. Several studies have reported that inflammation precedes and accompanies pathological angiogenesis as evidenced by increased vascular permeability, monocytes/macrophages and neutrophils recruitment at angiogenic sites [23,190]. During inflammatory processes, newly formed vessels supply inflamed tissue with oxygen and nutrients as well as facilitate the transport of inflammatory cells. 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) [56]. VEGF-mediated endothelial PAF synthesis occurs via a remodelling 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) [230]. Furthermore, we have demonstrated that in bovine aortic

endothelial cells (BAEC), VEGF-A_{165}-mediated PAF synthesis implies the dual activation of PLC-γ/PKC/p42/44 MAPK and MLK/MKK-3,-6/p38 MAPK signalling pathways whereas phosphatidyl inositol-3-phosphate kinase (PI3K) activation is not required [274]. Therefore, based on our previous observations with regards to VEGF-A_{165} and on the 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.

Our data demonstrate that the angiopoietins constitute a second class of tyrosine kinase receptor ligands capable of mediating endothelial PAF synthesis. In the present study, we demonstrate that both Ang1 and Ang2 induce endothelial PAF synthesis in a time-dependent manner, 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 [56], both angiopoietins induce a rapid, progressive, and sustained endothelial PAF synthesis (maximal within 4 hours) whereas VEGF-A_{165} induces a rapid and transient synthesis of PAF (maximal within 15 minutes) [56]. In activated endothelial cells, acute PAF synthesis is mediated through the remodelling pathway and can occur in a very early (2 - 5 minutes), early (10 - 40 minutes), or delayed (4 - 8 hours) [197] 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. We demonstrate herein that Ang1 and Ang2 are both capable of inducing PAF synthesis in BAEC whereas in a previous study, we reported that angiopoietins did not induce PAF synthesis in HUVEC [149]. At first glance, this may appear contradictory, but it may in fact be indicative of tissue specificity. Indeed, we have previously observed that VEGF-A_{165} induces a more robust endothelial PAF synthesis in BAEC as compared to HUVEC [230]. Therefore, the effect of angiopoietins on PAF synthesis is more evident in aortic than in venous endothelial cells.

The maximal PAF synthesis observed at 4 hours 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 (PD98059) to completely block VEGF-A_{165}-mediated endothelial PAF synthesis resides in its ability to prevent PLA_2 activation [274]. 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 [281], 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 [274]. 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 ([282] for review). As mentioned above, the remodelling 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 [226,283,284]. 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 $cPLA_2$ [282]. 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

prevent but even slightly increased EC PAF synthesis by both Ang1 and Ang2 at 4 hours. We have previously reported that $sPLA_2$-V is implicated in VEGF-A_{165}-mediated EC PAF synthesis [230] 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 of 10^{-4} M, 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 [230], 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 ([197] 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 [285-288], 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%. 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 minutes of stimulation with Ang1 and to a lesser extent with Ang2. The reduced response observed with Ang2 may be related to its less intense activation of Tie2 and may also explain why Ang2 is less potent at mediating PAF synthesis than Ang1. Figure 12 represents a summary of the purported intracellular signalling pathways implicated in angiopoietin-mediated PAF synthesis.

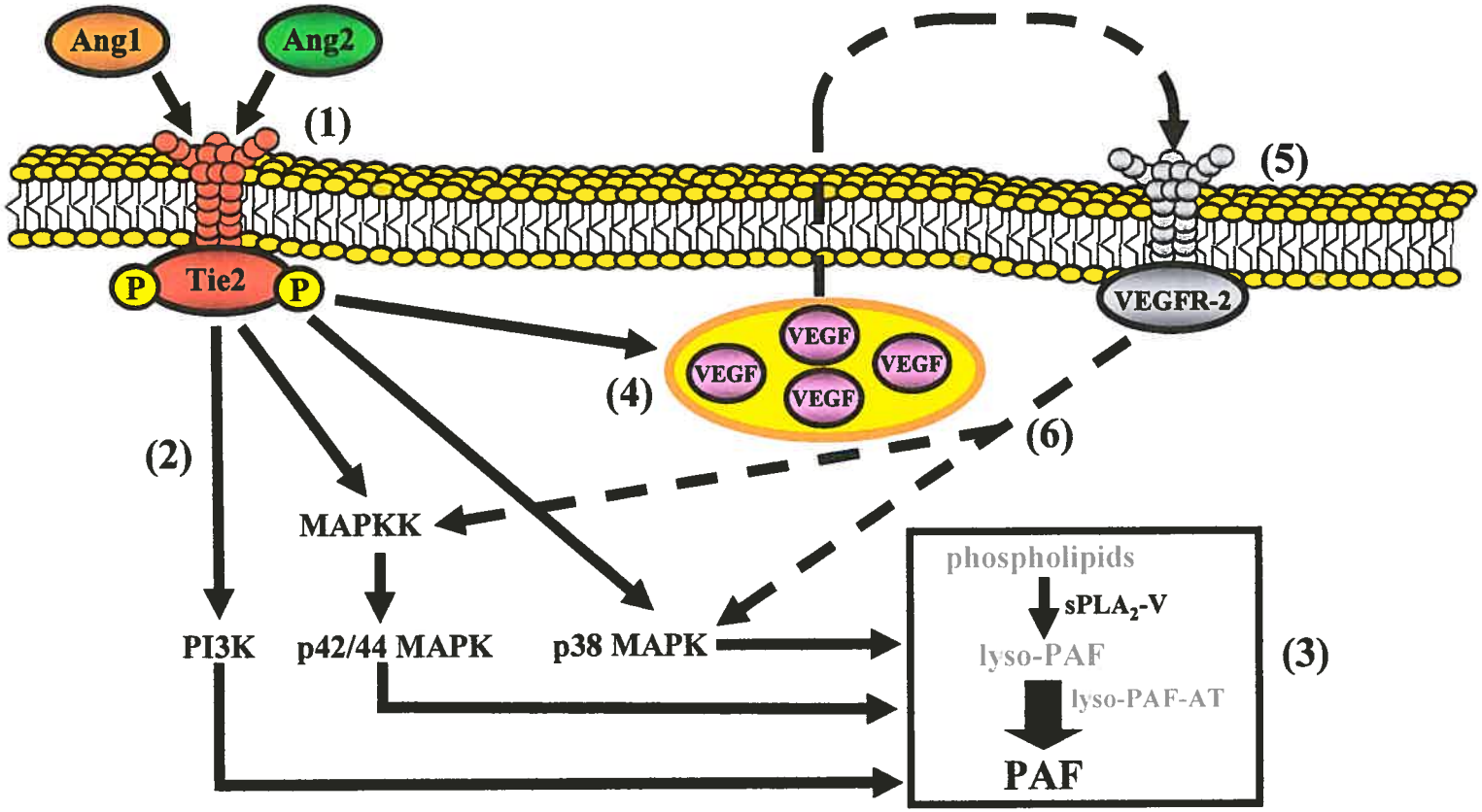

Figure 12: Proposed mechanism by which angiopoietins mediate endothelial PAF synthesis. Stimulation of Tie2 by Ang1 or Ang2 (1) activates PI3K, p42/44 MAPK, and p38 MAPK pathways (2). These intracellular signalling pathways, through the action of $sPLA_2$-V, induce endothelial PAF synthesis (3). Concurrently, 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 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.

4.3 Angiopoietin-induced P-selectin translocation in BAEC is calcium-dependent

We have reported that VEGF-A_{165} induces P-selectin translocation through endogenous PAF synthesis in HUVEC [65]. We have also recently observed that both Ang1 and Ang2 can promote P-selectin translocation in absence of PAF synthesis in HUVEC [149]. However, in this study, we observed that Ang1 and Ang2 as VEGF-A_{165} can mediate PAF synthesis in BAEC. We therefore sought to dissect the signalling pathways implicated in angiopoietin-mediated P-selectin translocation and determine the contribution of endogenous PAF in BAEC using VEGF-$_{A165}$ as a positive control. Herein, we report that both angiopoietins mediate 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 from Weibel-Palade bodies (WPB) upon stimulation with various inflammatory mediators as rapidly as 2 minutes after stimulation with a peak attained within 10 minutes [251,252] whereupon P-selectin enzymatic cleavage or endocytosis and is recycled back into WPB [248].

In the current study, we demonstrate that angiopoietin-mediated endothelial P-selectin translocation requires PLC and PKC activation. Indeed, treatment of BAEC with selective inhibitors of PLC-γ (U73122) or PKC (Calphostin C) prior to stimulation with Ang1 or Ang2 reduced P-selectin translocation. Similarly, VEGF-A_{165}-mediated P-selectin translocation is also dependent on the activation of PLC-γ and PKC. PLC-γ is an important regulator of calcium signalling and PKC,

immediately downstream and is implicated in p42/44 MAPK activation which in turn regulates endothelial PAF synthesis. Taken together, these observations prompted us to investigate calcium signalling and intracellular signalling pathways leading to endothelial PAF synthesis.

Since we and others have reported that Ang1 and Ang2, upon binding to Tie2, have been shown to activate p42/44 MAPK, p38 MAPK, and PI3K intracellular signalling pathways [127,146,162,164,166,278] leading to a rapid and sustained synthesis of PAF in BAEC as reported above, we wished to determine whether these pathways and PAF were implicated in angiopoietin-mediated P-selectin translocation. In the current study, we demonstrated that the aforementioned pathways are not implicated in angiopoietin-mediated endothelial P-selectin translocation and neither is endothelial PAF. On the other hand, VEGF-A_{165}-mediated translocation requires the activation of p38 MAPK and p42/44 MAPK, both capable of mediating endothelial PAF synthesis and PI3K which, although activated, has been shown not to increase PAF synthesis [274]. When we pretreated BAEC with selective PAF receptor antagonists prior to stimulation with VEGF-A_{165}, we observed a reduction in P-selectin translocation, although not as pronounced as what we had previously reported in HUVEC [65]. Taken together, our data demonstrates that the 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.

The translocation of P-selectin and constituents of WPB requires the movement of the WPB from the cytoplasm to the cell membrane and the fusion of these vesicles with the plasma membrane. Increased levels of cytosolic free Ca^{+2} concentration ($[Ca^{2+}]_i$) levels have been implicated in the mechanism of exocytosis for a number of agonists including thrombin and histamine [289]. The cellular responses to increased $[Ca^{2+}]_i$ are most likely mediated through calmodulin and small GTP-binding proteins [290-292]. In our current study, treatment of BAEC with an intra- and extracellular calcium chelator (BAPTA-AM) or a calmodulin inhibitor (W-7) prior to stimulation with Ang1 or Ang2 completely abrogated P-selectin translocation. Furthermore, P-selectin translocation was absent in cells treated with Ang1 or Ang2 in calcium-free DPBS. Together, these data confirm the importance of Ca^{2+} in P-selectin translocation mediated by angiopoietins. It has been established that elevation of Ca^{2+} over basal levels (10^{-7} M) is required for regulated exocytosis [293,294] and that this elevation may be due to influx of calcium across the plasma membrane [295], from internal stores, or both [296,297]. The endoplasmic reticulum (ER) is the best characterized Ca^{2+} store in mammalian cells [298] and release of Ca^{2+} from the ER can be triggered by activation of inositol triphosphate (IP_3) [299] which can also modulate calcium release from the Golgi complex [300]. In our current study we show that PLC-γ, which can act on PIP_2 to produce IP_3 and hence trigger Ca^{2+} release from intracellular stores, regulates angiopoietin-mediated endothelial P-selectin. Once released, calcium may form a complex with calmodulin. This complex has been shown to interact with a small GTP-binding protein, Ral, in a calcium-dependent

manner and play an important role in regulating WPB exocytosis in EC [289]. In fact, we observed that chelation of intra- and extracellular calcium completely abrogated endothelial P-selectin translocation. Furthermore, we observed that the release of Ca^{2+} from intracellular pools was insufficient to support endothelial P-selectin translocation in an extracellular Ca^{2+}-free environment. Using another approach, namely inhibiting the formation of the Ca^{2+}/calmodulin complex with W-7 and this, regardless of intra- or extracellular levels of calcium, reduces translocation to levels below control values confirming the importance of calcium in P-selectin translocation. Similarly, calcium also contributes to mediate VEGF-A_{165}-induced endothelial P-selectin translocation. Taken together, these observations demonstrate the critical role of calcium in VEGF-A_{165} and angiopoietin-mediated endothelial P-selectin translocation. In Figure 13, a summary of the signalling pathways regulating both VEGF-A_{165} and angiopoietin-mediated P-selectin translocation in BAEC is presented.

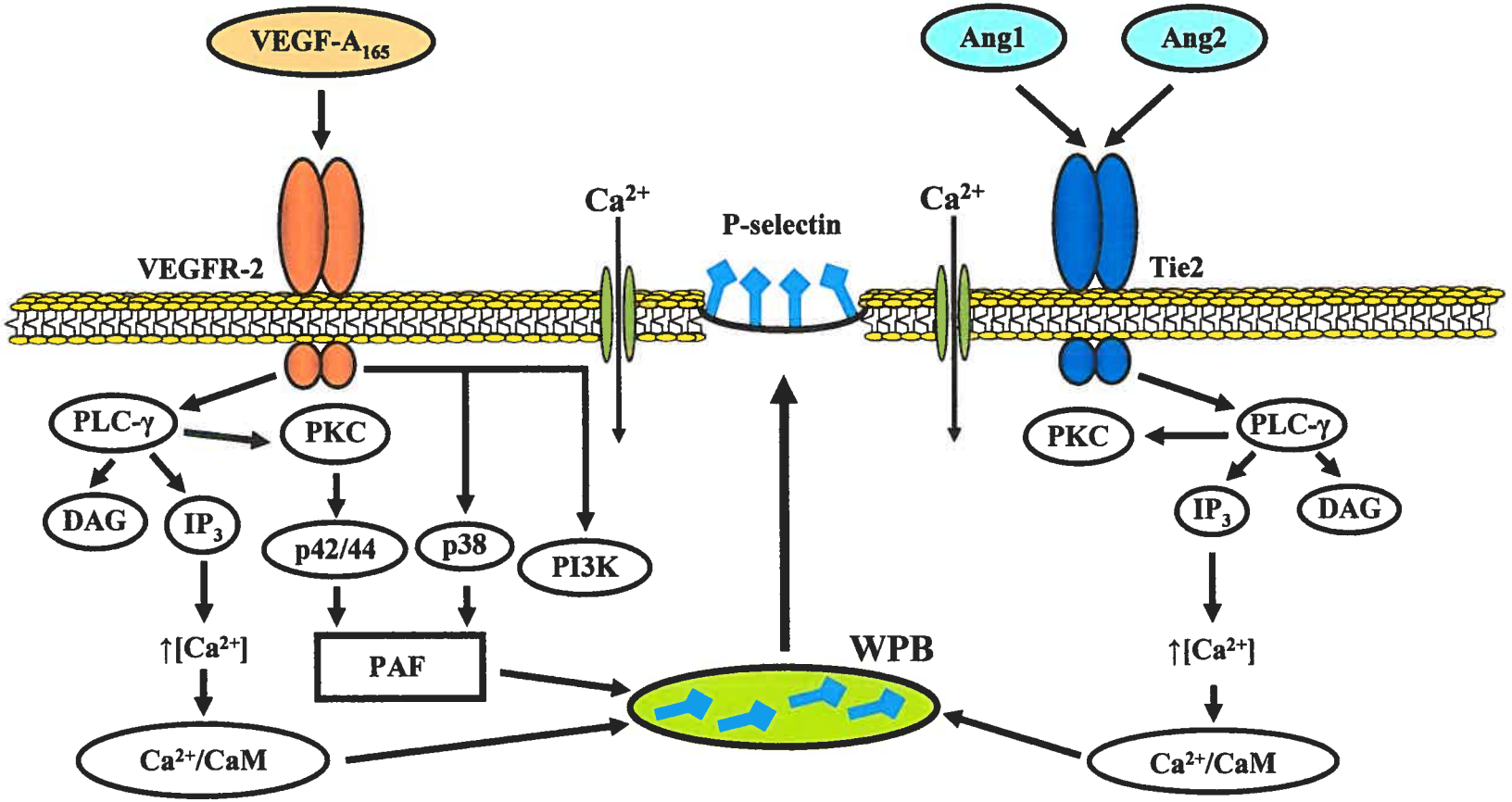

Figure 13: Proposed mechanisms for VEGF-A_{165} and angiopoietin-mediated endothelial P-selectin translocation. Stimulation of VEGFR-2 by VEGF-A_{165} activates PLC-γ and PKC leading to the activation of p42/44 and p38 MAPKs which in turn mediate endothelial PAF synthesis. In addition, PLC-γ mediates calcium release from intracellular stores through IP_3, leading to Weibel-Palade body exocytosis and P-selectin translocation to the cell surface. Endothelial P-selectin translocation also requires cytosolic calcium. Angiopoietin-mediated P-selectin translocation also requires calcium and PLC-γ but contrary to VEGF-A_{165}, angiopoietin-mediated P-selectin does not require endogenous PAF synthesis.

4.4 Angiopoietins are inflammatory mediators

Based on our current observations as well as what we have reported in 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 [149]. 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 [301] and *in vitro* it reduces the basal activation of vascular endothelial cadherin (VE-cadherin) and β-catenin, components of intercellular junctions [189], concomitantly with a reduction of VEGF-mediated endothelial cell permeability [185,186]. 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 addition, Ang1 mediated a reduction of VEGF-induced leukocyte adhesion onto HUVEC and E-selectin expression [148] and thrombin-mediated neutrophil adhesion onto EC [185]. Upon initial review, these results may appear contradictory to what we report herein as well as what we have previously

demonstrated [149] but both of the above studies [148,185] were performed for extended periods of time that are well in excess of the timeframe of P-selectin activity.

One would expect Ang2 to readily induce PAF synthesis if one considers its ability to disrupt vessel integrity. Surprisingly, until recently, the role of Ang2 in inflammation was unknown. In addition to our recent demonstration that Ang2 (as well as Ang1) promotes neutrophil PAF synthesis, endothelial P-selectin translocation, and neutrophil adhesion onto EC [149], Ang2 has been shown to promote vascular leakage *in vivo* without displaying the full features of a true proinflammatory mediator since it only weakly promoted leukocyte migration [150]. The authors of the aforementioned study observed that administration of Ang1 did not inhibit VEGF-induced vascular permeability. Citing our work [149], Roviezzo *et al.* [150] postulate that the presence of other inflammatory cells expressing Tie2 within the airpouch environment modified the response to Ang1 and thus Ang1 activation of neutrophil Tie2 receptors increases their adherence and release of PAF. Furthermore, Ang2 has been shown to be stored exclusive of P-selectin and rapidly released from WPB upon stimulation with PMA and calcium ionophores but not with VEGF or Ang1 [152]. This ability to be rapidly released upon stimulation suggests that Ang2 is an important component of rapid vascular responses such as inflammation. Taken together, these observations demonstrate that the angiopoietins should be considered as acute proinflammatory mediators.

4.5 Perspectives

In light of our observations with respect to the potential proinflammatory properties of Ang1, one may be prompted to conclude that our observations are partly due to our experimental model. Indeed, as mentioned above, previous studies have reported on the anti-inflammatory nature of Ang1 using other cell types (HUVEC) [148,185,186,189] or its vessel-stabilizing nature in animal models [123,137,301-303]. Our data suggests tissue specificity with respect to the proinflammatory abilities of Ang1 since we have demonstrated that Ang1 can induce P-selectin translocation but not PAF synthesis in another cell type, namely HUVEC [149]. The ability of BAEC to produce greater amounts of PAF than HUVEC has been documented by our laboratory [230] and prompted us to select this experimental model. Thus, to better define the role of Ang1 in endogenous PAF synthesis, *in vivo* studies should be performed.

In order to verify our *in vitro* data, models of localized inflammation could be utilized. One potential approach is the murine air pouch [150,304] where sterilized air is injected subcutaneously into the back of the animal six (6) and three (3) days prior to the injection of the desired substance into the air pouch. Ang1, for example, could be injected into the air pouch for various time periods, the pouch exudates collected and screened for the presence of various inflammatory mediators. Due to its rapid degradation, endothelial PAF could not be directly measured and thus its role could be delineated by the use of PAF receptor antagonists. An increase in vascular permeability brought upon by an increase in endothelial PAF synthesis could be

observed by quantifying Evans blue exudation into surrounding tissue. In addition, specific pathway inhibitors and Tie2 blocking antibodies could, for example, be injected into the air pouch to delineate the signalling pathways implicated. Similarly, the mouse paw edema model [150] may prove to be an attractive model with which to determine the role of Ang1 in endogenous PAF synthesis *in vivo*. In this model, animals receive subplantar administrations of various agents and paw volume is determined using a hydroplethysmometer modified for small volumes [150]. PAF synthesis would be observed through an increase in limb volume due to local edema brought upon by an increase in vascular permeability. In addition to using pharmacological antagonists and inhibitors, our laboratory has access to PAF-R and $sPLA_2$-V null mice which could in turn be utilized to determine the effect of Ang1 on endogenous PAF synthesis.

Recent studies have reported on the proinflammatory role of Ang2 *in vivo* [150,305,306] and these observations, at first glance, seem to be in line with our *in vitro* work. All of the animal studies proposed above to investigate the role of Ang1 in endogenous PAF synthesis should be performed with Ang2 as well.

The ability to measure endothelial P-selectin translocation *in vivo* proposes to be technically challenging. Initially, a flow chamber apparatus could be used to assess the ability to label endothelial P-selectin in a dynamic (non-static) environment. In an intact animal, however, two-photon microscopy, because of its resolving power, may allow the observation of P-selectin translocation *in vivo*. In such an experiment,

an artery (ie. carotid) could be isolated from surrounding anatomical structures in an anaesthetized animal. A P-selectin antibody coupled to a chromophore could then be injected upstream of the area of interest to allow it to bind to EC lining the artery. Promoters of P-selectin translocation such as PMA, thrombin, and angiopoietins could then be injected into the animal.

Another potential study would entail the determination of the role of Tie1 in endogenous PAF synthesis and endothelial P-selectin translocation. There is some evidence that under certain circumstances, Tie1 forms a complex with Tie2 [132,133] and in addition, Tie1 may also signal through PI3K/Akt [135]. However, little is known about the ability of Tie1 to modulate Tie2 activity. On another scale, *ex vivo* experiments could also be conducted in an organ bath using isolated vessels to explore the role of angiopoietins in modulating acute inflammatory responses.

Our data demonstrate *in vitro* the ability of angiopoietins to behave as proinflammatory mediators. However, the inflammatory response is a complex process and a better understanding is complemented through *in vivo* studies. Our work has delineated the intracellular signalling pathways implicated in angiopoietin-mediated endothelial PAF synthesis and P-selectin translocation but animal studies are necessary to validate these observations.

5.0 Conclusion

In light of the objectives of this study, we can conclude the following:

(1) 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}.

(2) Angiopoietin-mediated endothelial PAF 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, it is partly regulated by a redistribution of endogenous VEGF to the cell surface membrane which may subsequently potentiate endothelial PAF synthesis.

(3) Ang1 and Ang2 induce a rapid and transient translocation of P-selectin dependent on calcium and modulated through PLC-γ activation. In addition, contrary to what we have observed with respect to VEGF-A_{165}, angiopoietin-mediated P-selectin translocation does not require the synthesis of endogenous PAF.

Taken together, our results demonstrate that the angiopoietins, like VEGF-A_{165}, constitute a second family of angiogenic growth factors capable of promoting proinflammatory events, namely endothelial PAF synthesis and P-selectin translocation. This study allowed us to gain a better understanding of the

intracellular mechanisms mediating angiopoietin-induced endothelial PAF synthesis and P-selectin translocation. By identifying and delineating the intracellular mechanisms governing endothelial PAF synthesis and P-selectin translocation mediated by angiopoietins *in vitro*, we establish a platform for future studies and development of novel anti-inflammatory and/or antiangiogenic therapies.

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Appendix

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

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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,

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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

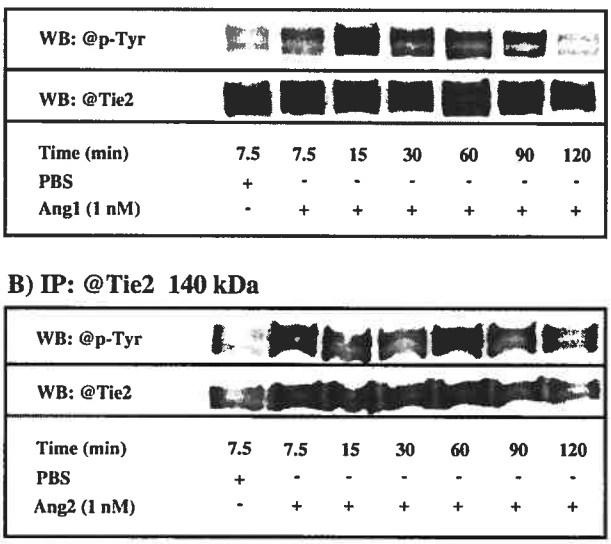

Fig. 1. Activation and expression of Tie2 in BAEC. Confluent BAEC were treated with Ang1 (A), or Ang2 (B) for up to 2 h. Cell lysates were prepared and immunoprecipitated (IP) with rabbit polyclonal anti-mouse Tie2 IgG from 500 μg of lysate. Following resolution by SDS–PAGE and transfer, PVDF membranes 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

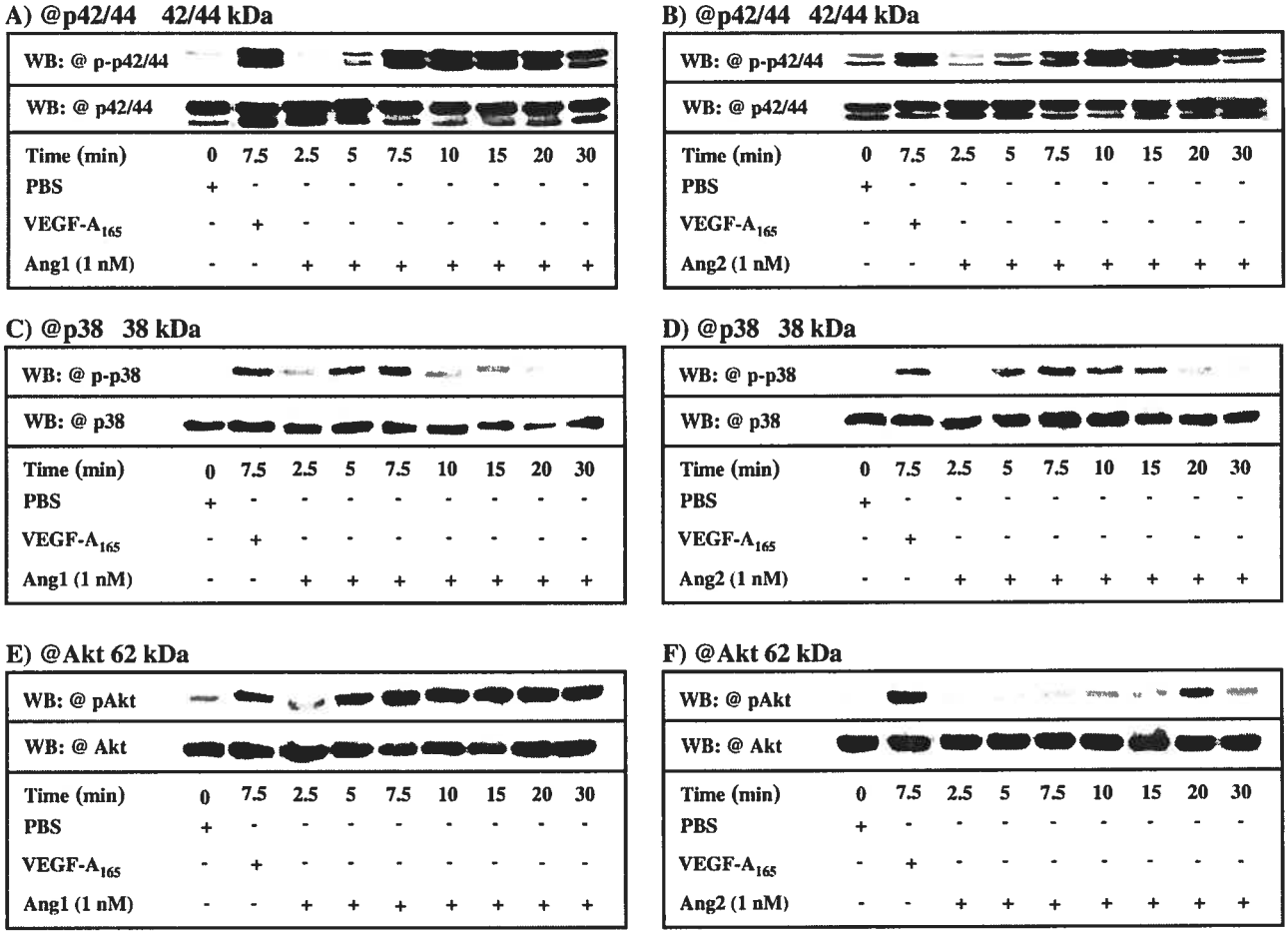

Fig. 2. Ang1 and Ang2 activate p38 MAPK, p42/44 MAPK, and PI3K pathways. Confluent BAEC were stimulated with Ang1, or Ang2 (1 nM) for up to 30 min. BAEC were also stimulated with VEGF-A_{165} for 7.5 min as positive control. Cell lysates equivalent to 100 μg total proteins were loaded in each lane. Signalling 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).

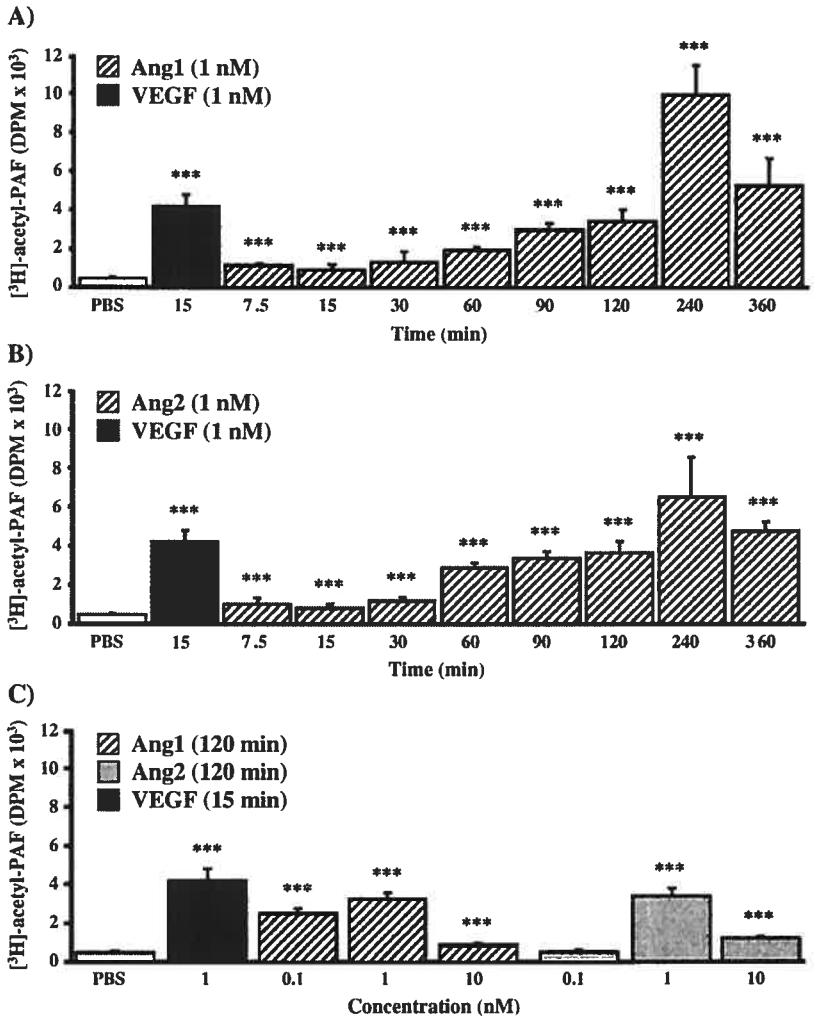

Fig. 3. Ang1 and Ang2 are both capable of inducing endothelial PAF synthesis in a time and concentration-dependent manner. Confluent BAEC were stimulated with Ang1 or Ang2 (1 nM) for time periods ranging from 7.5 to 360 min in the presence of [^{3}H]-acetate. As positive control BAEC were treated with 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

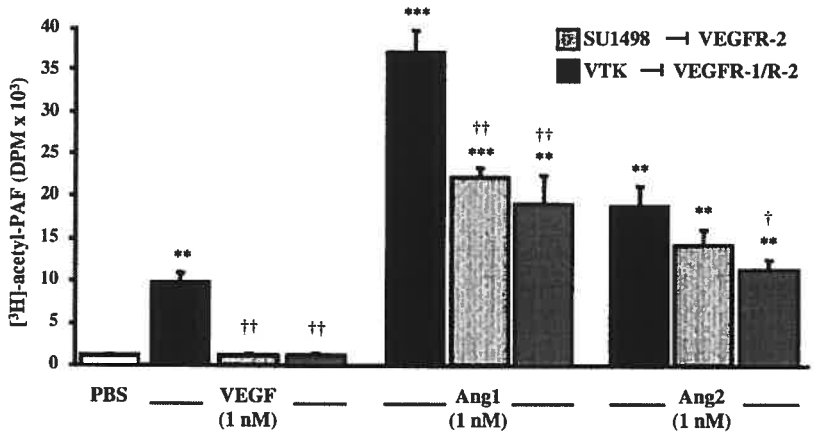

Fig. 4. Pretreatment with VEGF receptor (VEGFR) inhibitors attenuates angiopoietin-mediated endothelial PA synthesis at 4 h. Confluent BAEC were pretreated with a selective VEGFR-2 inhibitor (SU1498; 5 μM) or a VEGFR-1/R-2 inhibitor (VTK; 10 μM) for 15 min prior to stimulation with Ang1 or Ang2 (1 nM) 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

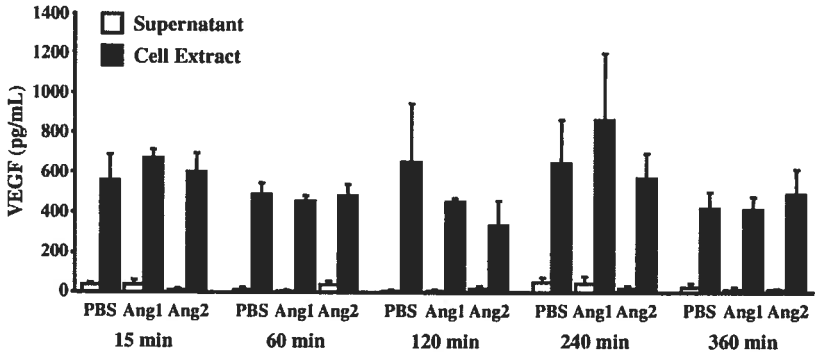

Fig. 5. Endothelial distribution of VEGF upon stimulation with angiopoietins. Confluent BAEC were stimulated in serum-free DMEM with Ang1 or Ang2 (1 nM) for up to 6 h. Supernatants were collected, concentrated and VEGF protein quantified by sandwich ELISA. Cell membranes were gently scraped in cold PBS (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.

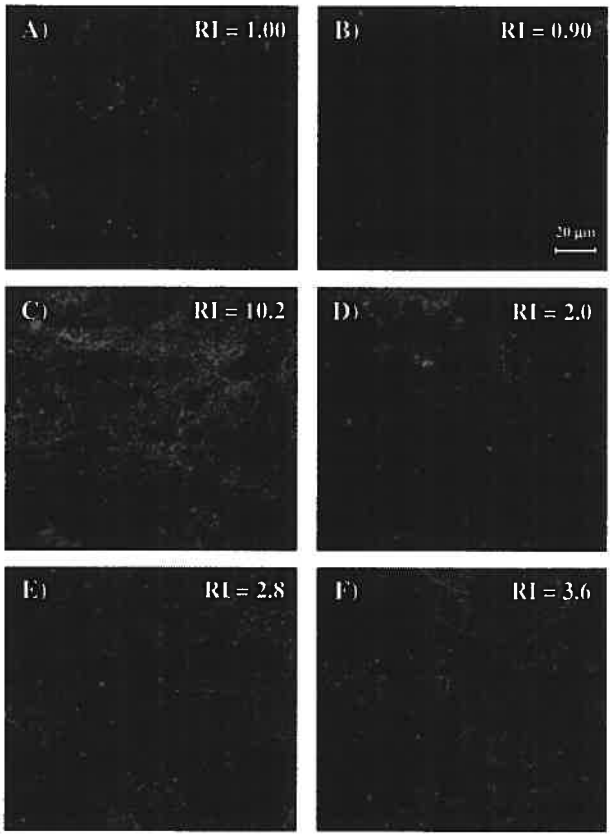

Fig. 6. Treatment of BAEC with angiopoietins induces a relocalization of endogenous VEGF to the cell surface membrane. Subconfluent BAEC were grown on gelatin-coated glass slides, rinsed with PBS, left untreated (A and B) or treated with Ang1 or Ang2 (both 1 nM) for 7.5 min (C and E, respectively) or 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

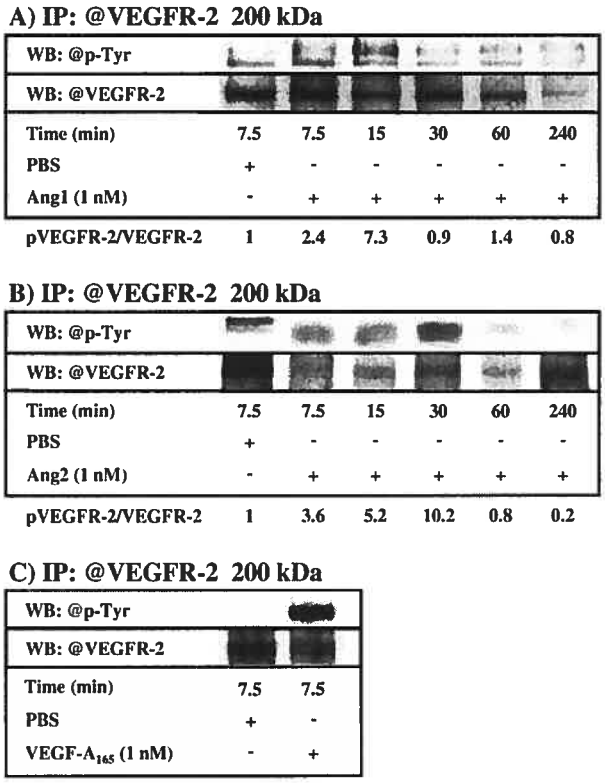

Fig. 7. Treatment of BAEC with Ang1 or Ang2 promotes VEGFR-2 phosphorylation. Confluent BAEC were stimulated with Ang1 or Ang2 (1 nM) for 7.5 min to 4 h. Cell lysates (1 mg) were prepared and immunoprecipitated (IP) with rabbit polyclonal anti-mouse VEGFR-2 IgG. Following resolution by SDS–PAGE and transfer, 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.

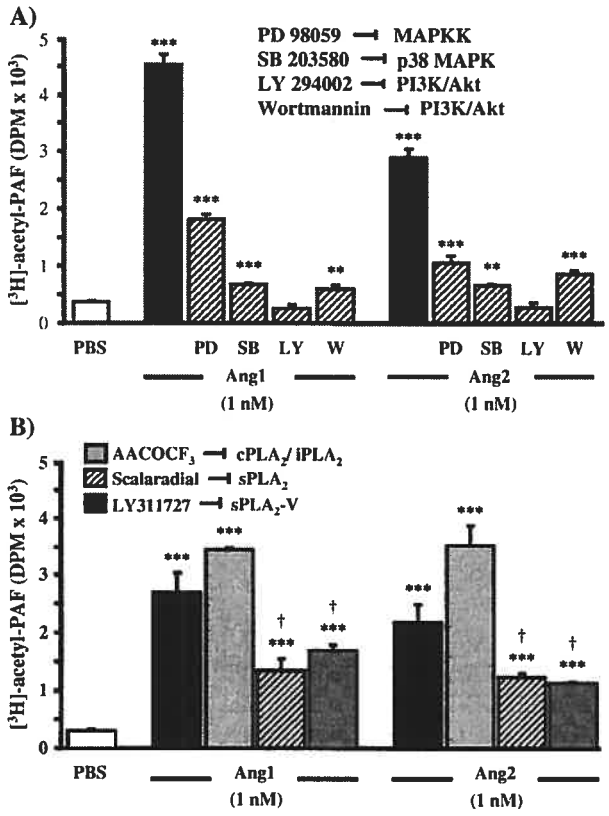

Fig. 8. Angiopoietin-mediated endothelial PAF synthesis requires the activation of p38 MAPK, p42/44 MAPK, and PI3K/Akt signalling pathways and $sPLA_2$-V. (A) Confluent BAEC were pretreated with either PD98059 (10 μM), SB203580 (10 μM), LY294002 (5 μM), or Wortmannin (500 nM) 15 min prior to 4 h of stimulation 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

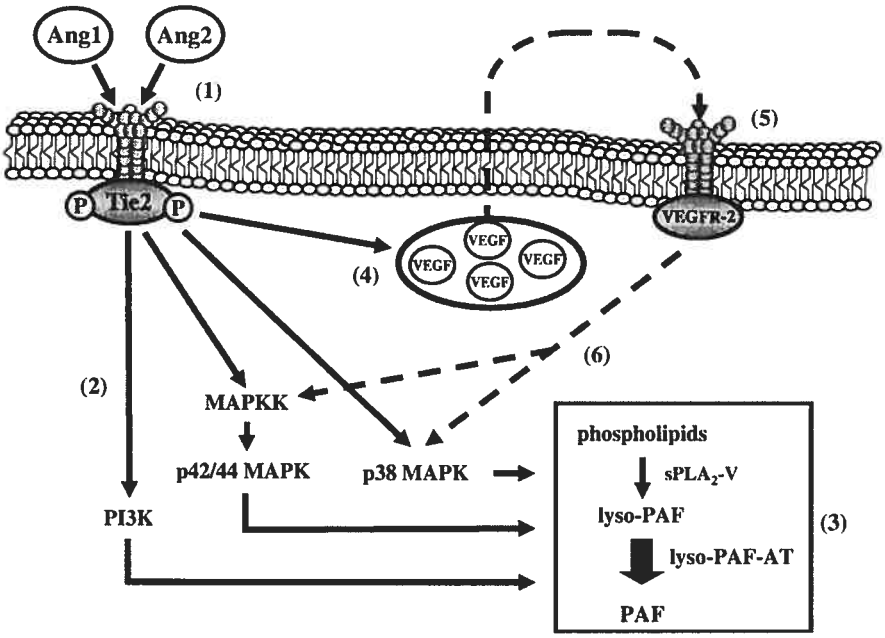

Fig. 9. Proposed mechanism by which angiopoietins mediate endothelial PAF synthesis. Stimulation of tyrosine kinase Tie2 receptor by Ang1 or Ang2 (1) activates PI3K, p42/44 MAPK, and p38 MAPK pathways (2). These intracellular signalling pathways, through the action of $sPLA_2$-V, induce endothelial PAF 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

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