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Review

Phosphoinositide 3-kinase signalling in the vascular system

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Phosphoinositide 3-kinases (PI3Ks) are protein and lipid kinases activated by different classes of membrane receptors, including G-protein coupled and tyrosine kinase receptors. Several lines of evidence have uncovered specific roles for distinct PI3K isoforms in the vascular system in both physiology and disease. The present review will summarize and discuss the most recent advances regarding PI3K-Akt signalling in endothelial cells, vascular smooth muscle cells, platelets, and inflammatory cells involved in the atherosclerotic process. Of interest, the development of novel isoform-selective PI3K inhibitor drugs offers a unique opportunity to selectively and differentially target PI3K-driven pathways in the vascular system and may give rise to new strategies for the treatment of cardiovascular diseases.

1. Introduction

Phosphoinositide 3-kinases (PI3Ks) are a conserved family of enzymes characterized by dual protein and lipid kinase activity. Members of this family differ in protein structure, expression, regulation, and substrate specificity, but all share a common catalytic function: they phosphorylate the D3 hydroxyl group of membrane phosphatidylinositols (PtdIns) upon receptor tyrosine kinase (RTK) and G-protein-coupled receptor (GPCR) stimulation or Ras activation. PI3K isoenzymes are currently grouped into three classes.[1] Class I PI3Ks are heterodimers composed of a catalytic (p110α, β, δ, and γ) and a regulatory subunit (p85 or p101 family) and are the only PI3Ks that phosphorylate PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. Although PI3Kα and β are ubiquitous and abundantly expressed in the vascular system, the expression of PI3Kδ and γ is mainly restricted to leucocytes. However, the expression of PI3Kγ has also been recently described in several cardiovascular tissues, including heart, vasculature, and platelets. Class II PI3Ks produce PtdIns(3)P from PtdIns and has also been reported to contribute to PtdIns(3,4)P_2 production. Three different class II monomers have been identified: the ubiquitous PI3K-C2α and C2β, and the liver specific PI3K-C2γ. Vacuolar protein sorting 34 (Vps34), the only member of class III, generates only PtdIns(3)P and is ubiquitously expressed. With respect to vascular biology, class I PI3Ks are the best characterized isoforms, whereas less is known about class II and class III.

PI3K signalling is tightly regulated by lipid phosphatases, which remove the phosphate groups added by PI3Ks. At least three lipid phosphatases play this role and all are expressed in vascular tissues. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and myotubularin act as 3-phosphatases, degrading, respectively, PtdIns(3,4,5)P_3 and PtdIns(3)P, whereas the Src-homology 2 (SH2)-containing inositol phosphatase exerts a 5-phosphatase activity.

PI3Ks activate diverse cellular targets carrying the pleckstrin homology domain, a lipid-binding domain present in all primary effectors of the PI3K signalling system. By binding phosphorylated phosphatidyl-inositols, this domain facilitates the recruitment of downstream effectors to the plasma membrane. The prototype enzyme activated by PI3Ks is protein kinase B (PKB/Akt), a serine–threonine kinase. Three different Akt isoforms are known: Akt1, Akt2, and Akt3. Among them, Akt1 appears to be the enzyme mostly relevant to cardiovascular functions.[2] Other known PI3K downstream effectors with a potential involvement in the cardiovascular system include glycogen synthase kinase 3 (GSK3), Raf, forkhead box transcription factors (FOXOs), RhoA, and phospholipase C (PLC) (reviewed by Hirsch *et al.*[3]).

The present review will focus on the specific functions of PI3K signalling in the vascular system in normal physiology and disease. In particular, we will discuss the role of these enzymes and their downstream effectors in the vascular wall, including endothelium, vascular smooth muscle, platelets, and atherosclerotic plaques.

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2. Phosphoinositide 3-kinase signalling in endothelial cells

The PI3K/Akt pathway is involved in prototypical endothelial functions such as the regulation of vascular tone, angiogenesis, control of adhesion, and recruitment of leucocytes to the vessel wall (*Figure 1*). In particular, recent studies have begun to uncover the role of single PI3K isoforms in nitric oxide (NO) synthesis, endothelial-leucocyte interaction, endothelial progenitor cell (EPC) biology, and angiogenesis.

2.1 Nitric oxide synthase

PI3K and Akt are important positive regulators of endothelial nitric oxide synthase (eNOS), which generates NO through the NADPH-dependent oxidation of L-arginine. Among the different stimuli leading to NO release from endothelial cells, those implicating PI3K signalling include humoral factors [e.g. insulin-like growth factor-1 (IGF-1),[4] insulin,[5,6] sphingosine-1-phosphate (S1P),[7,8] vascular endothelial growth factor (VEGF)[4,9–12]], and shear stress.[13,14] Although humoral factors activate PI3K and Akt through binding to cognate endothelial receptors (GPCRs or RTKs), shear stress has been suggested to act via $\alpha_1\beta_1$ integrin, which functions as a mechanic sensor.[14]

The function of eNOS is tightly regulated at several levels, including the transcriptional, post-transcriptional, and post-translational (reviewed elsewhere[15,16]). The latter involves eNOS phosphorylation on different residues [by different kinases, including protein kinase A (PKA), AMP-activated protein kinase (AMPK), and protein kinase C (PKC)] and protein–protein interactions (e.g. with calmodulin, caveolin-1, heat shock protein 90). Following PI3K stimulation, activated Akt phosphorylates eNOS on Ser-1177, enhancing both basal and stimulated eNOS enzyme activity and thus NO release.[4,9–11] Such event is eNOS-specific, since the other NOS isoforms [neuronal and inducible NOS (nNOS, iNOS)] are not functionally affected by Akt.[10] Of note, Ser-1177 is not a site of exclusive phosphorylation by Akt, since wortmannin does not completely block its ligand-induced phosphorylation. Other kinases phosphorylating Ser-1177 include AMPK[17] and PKA.[18]

The regulation of eNOS by Akt has been directly shown *in vivo* and *ex vivo*. Infection of arterial endothelial cells with a viral vector encoding for a constitutively active Akt is associated with local NO-dependent vasodilation and increased blood flow, whereas infection with a vector encoding for a dominant-negative Akt blunts acetylcholine-dependent NO release and subsequent vasorelaxation.[19] By delivering a phosphomimetic form of eNOS (S1179DeNOS) to the endothelium of isolated carotid arteries from eNOS-deficient mice, Fulton and coworkers were able to reconstitute basal and stimulated NO release, displaying the importance of the Ser-1177 residue in endothelial-dependent vasomotion.[20] Moreover, a rapid local phosphorylation of Akt and eNOS has been shown in rats upon penile erection, whereas the administration of wortmannin and LY294002 abolished Akt and eNOS phosphorylation and attenuated erection.[21] Interestingly, among Akt isoforms, Akt1 may be most extensively involved in endothelial cells, since Ackah *et al.*[22] have reported that the selective loss of Akt1 is associated with reduced eNOS phosphorylation, NO release, and angiogenesis.

The precise molecular mechanisms leading to enhanced eNOS activity upon Akt phosphorylation are only partially understood. It is well established that the Akt-eNOS interaction specifically occurs at the plasma membrane and that Ser-1177 phosphorylation renders eNOS significantly more sensitive to the levels of intracellular calcium.[9,10] Since truncation of eNOS at Ser-1177 leads to increased enzymatic activity, a proposed model is that Ser-1177 phosphorylation may act by removing autoinhibition by the COOH-terminal tail of the protein.[17] Alternatively, the

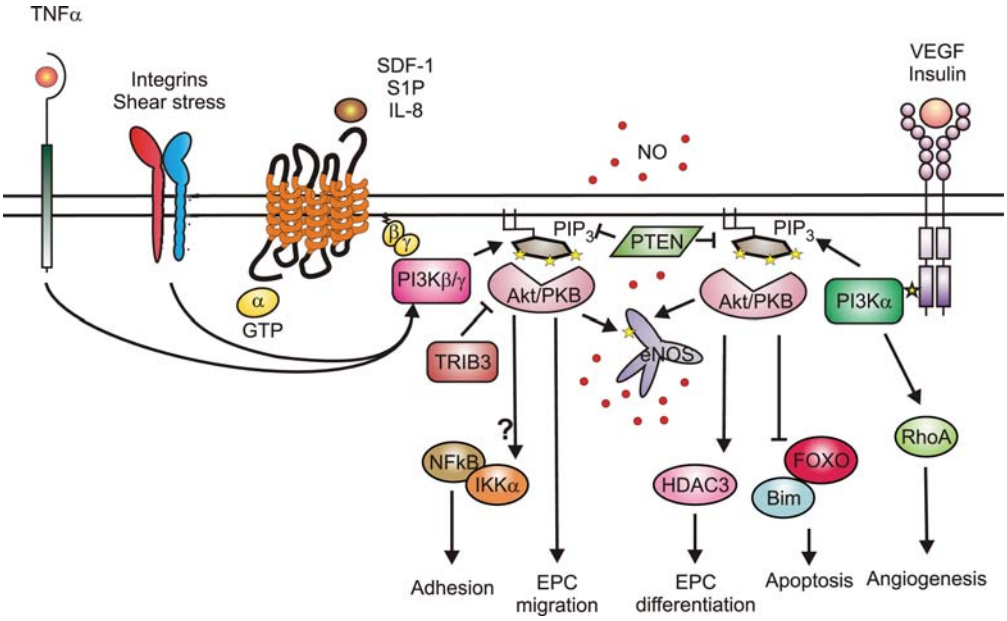

Figure 1 In endothelial cells, phosphoinositide 3-kinases are activated downstream of several receptors, including G-protein-coupled receptors (e.g. chemokine receptors), tyrosine kinases (e.g. vascular endothelial growth factor receptors), integrins, and death receptors (e.g. TNFα receptor). In turn, phosphoinositide 3-kinase signalling promotes nitric oxide release (through endothelial nitric oxide synthase phosphorylation), angiogenesis (through RhoA), endothelial progenitor cell (EPC) recruitment, and cell viability.

phosphorylation of eNOS could interfere with the structural interaction between its COOH-tail and the calmodulin-autoinhibitory loop and/or influence the interaction of eNOS with other proteins.[10,15]

Different lines of evidence have suggested that the action of Akt on eNOS can be independently modulated. For instance, proteins of the tribbles homologue 3 (TRIB3) group can selectively counteract the phosphorylation of eNOS by Akt. R84, a TRIB3 variant that causes Akt inhibition, is associated with reduced NO release from endothelial cells, representing a model of genetically determined disruption of the PI3K/Akt/eNOS pathway.[23] Furthermore, conditions of insulin resistance have also been shown to inhibit Akt phosphorylation on eNOS Ser-1177.[24] Although the precise molecular mechanisms underlying this phenomenon still lack a detailed description, the down-regulation or desensitization of the PI3K/Akt/eNOS pathway has been advocated as a potential mechanism underlying endothelial dysfunction and vascular disease correlated to insulin resistance.

2.2 Endothelial inflammation

Direct evidence has shown that endothelial PI3Kγ and PI3Kδ significantly contribute to E-selectin-dependent neutrophil-endothelial interaction in post-capillary venules, regulating cytokine-driven neutrophil rolling and migration. In fact, neutrophil attachment is significantly impaired on activated PI3Kγ or PI3Kδ-deficient (or selective PI3Kδ inhibitor-treated) endothelial cells and further compromised on a double PI3Kγ- and PI3Kδ-deficient endothelium, suggesting a cooperation between these two PI3K isoforms in this biological function.[25,26] Interestingly, PI3Kγ and PI3Kδ appear to affect the actual function and/or spatial distribution of E-selectin and not its raw expression levels.[26]

PI3K regulates endothelial-leucocyte interaction also in the context of ischaemia/reperfusion injury, which is dominated by oxidative stress, neutrophil adhesion, and *trans*-endothelial migration. In fact, Young *et al.*[27] have shown that pan-PI3K inhibition with wortmannin can successfully reduce neutrophil adhesion, infiltration, and reactive oxygen species (ROS) release in cardiac ischaemia/reperfusion injury in rats, resulting in protective cardiac effects. More recently, Doukas *et al.*[28] have reported that the selective inhibition of PI3Kγ and PI3Kδ (with compound TG100-115) during the reperfusion phase can substantially reduce final infarct size in rodents and pigs. In this study, the authors also examined the impact of TG100-115 on endothelial viability, ruling out a negative action of this compound on endothelial reparative proliferation. Selective PI3Kγ/δ inhibition may be even more effective than pan-PI3K blockade because the inhibition of PI3Kα and/or PI3Kβ in cells other than cardiomyocytes and leucocytes may actually determine unfavourable effects on tissue repair and revascularization.[29] The cardioprotective effect of TG100-115 may be largely attributed to reduced inflammatory signalling within the infarcted myocardium and to the related secondary tissue damage. Of note, Serban *et al.*[30] have recently reported that PI3Kγ and PI3Kδ are indeed involved in the VEGF-induced regulation of endothelial permeability downstream of H-Ras.

2.3 Endothelial progenitor cells

EPCs, present in the bone marrow and peripheral blood, are mononuclear precursor cells able to differentiate into mature endothelial cells (as shown by *in vitro* uptake of acetylated low-density lipoprotein, binding of lectin, and staining for endothelial markers such as VEGF receptor-2, CD31, and vascular endothelial cadherin).[31] PI3K and Akt are involved in the regulation of several EPC functions, including cell survival, homing, and differentiation into mature endothelial cells.

First, as in other cell types, the activation of PI3K/Akt signalling is pro-survival in EPCs. These effects are mediated by the inactivation of the pro-apoptotic forkhead transcription factors FOXO1, FOXO3a, and FOXO4, and by a reduced expression of the pro-apoptotic factor Bcl-2-interacting mediator of cell death (Bim).[32,33] Of note, both VEGF and statin therapy have been shown to increase EPC number through PI3K/Akt.[34] With respect to the homing process, EPCs migration into ischaemic tissues is affected by PI3K/Akt. Indeed, two major regulators of EPC trafficking, stromal cell-derived factor 1 (SDF-1/CXCL12) and VEGF, converge on PI3K/Akt/eNOS. Both the pharmacological inhibition of PI3K and the expression of a dominant-negative Akt result in reduced VEGF-controlled migration.[12] Vice versa, local Akt gene transfer to an ischaemic limb enhances homing of systemically administered progenitor cells.[35] Akt1 appears to be the key regulator of post-natal vasculogenesis. In fact, although knockout mice for Akt1 or Akt2 are viable, Akt1 knockout mice have reduced EPC mobilization in response to ischaemia, alongside with an impairment in ischaemic and VEGF-mediated angiogenesis, leading to severe peripheral vascular disease.[22]

Finally, also the differentiation of EPCs into mature endothelial cells is controlled by a VEGF receptor-2/PI3K/Akt pathway, which activates histone deacetylase 3 (HDAC3). In this process, HDAC3 mediates p53 deacetylation and hence p21 activation.[36] Additionally, the co-culture of EPCs with vascular smooth muscle cells (VSMCs) triggers EPC differentiation by enhancing the expression of endothelial markers [CD31 and von Willebrand factor (vWF)] and reducing progenitor ones (CD133 and CD34). Similarly, such co-cultures also result in Akt activation.[36]

Although all class I PI3Ks are expressed in EPCs, a pre-eminent role has been described for the PI3Kγ isoform.[37,38] In fact, PI3Kγ has been reported to modulate EPC homing and angiogenesis. Loss of PI3Kγ results in defective neovascularization and reperfusion after hindlimb ischaemia. Such findings are partly explained by reduced proliferation and enhanced apoptosis and partly by impaired integrin signalling in PI3Kγ-defective EPCs. Possibly, the role of PI3Kγ in EPCs may depend on both kinase-dependent and -independent mechanisms.

2.4 Angiogenesis

Several lines of evidence have indicated a role for PI3K signalling in blood vessel formation and repair. Mostly, the PI3K/Akt pathway has been studied in sprouting angiogenesis, which requires cell migration, vessel assembly, and tube formation. These mechanisms underlie the development of new blood vessels during embryonic development, tumour growth, and ischaemic conditions.[39]

VEGFs are prominent angiogenic regulators. Among the seven VEGF family members, established roles in angiogenesis have been shown for isoforms VEGF-A, VEGF-B, and placental growth factor, which promote endothelial

proliferation, migration, and tube formation (reviewed elsewhere[40]). VEGF actions are mediated by their binding to three specific RTKs (VEGFR-1/Flt-1, VEGFR-2/KDR/Flk-1, and VEGFR-3), which have been shown to activate PI3K/Akt signalling in endothelial cells.[41–45] Interestingly, the angiogenic actions of VEGFR-1 and VEGFR-2 require downstream eNOS phosphorylation and activation by Akt.[46] Since eNOS-deficient mice present defective VEGF-A-induced angiogenesis in hindlimb ischaemia[47] and impaired VEGF-A-dependent bone marrow mobilization of EPCs,[48] it appears that the PI3K-Akt-eNOS axis indeed constitutes a major determinant in post-natal angiogenesis at ischaemic sites. Not only can VEGF-A regulate angiogenesis *per se*, but it also affects vascular homeostasis through modulating the actions of distinct factors such as angiopoietins.[49,50] For instance, treatment of endothelial cells with VEGF-A elicits the shedding of angiopoietin receptors Tie1 and Tie2. Findley *et al.*[50] have recently reported that the shedding of Tie2 depends on PI3K/Akt both basally and upon VEFG-A stimulation. Noteworthy, this is the first study to report a role for the PI3K/Akt pathway in RTK shedding. Furthermore, it has been suggested that PI3K activity in angiogenesis may be controlled downstream of VEGFR occupancy by the availability of its substrate PtdIns(4,5)P_2. In fact, when PLCγ is activated, less PtdIns(4,5)P_2 is available for PI3Ks, thus counteracting angiogenic responses.[51] At early stages, the degree of VEGF-stimulated PI3K/Akt signalling has also been shown to determine angioblast differentiation towards vein or artery development.[52] Although prevalent extracellular signal-regulated kinase (Erk) signalling is associated with arterial fate, PI3K/Akt can block ErK activation, hence promoting venous differentiation, possibly via direct inhibition of Raf by Akt.[52]

A limited amount of data is available regarding the differential involvement of specific PI3K isoforms in angiogenesis. Yuan *et al.*[53] have reported that the endothelial-specific knockdown of class IA PI3Ks results in embryonic lethality, with evidence of vascular abnormalities such as microaneurisms, vessel enlargement, and haemorrhages. A recent work has uncovered a pivotal role for PI3Kα in regulating angiogenesis *in vivo*. Studies on mice expressing an ubiquitous or an endothelial cell-specific kinase-dead PI3Kα demonstrate that this enzyme is not required during the initial stages of vascular development, whereas it becomes strictly necessary for subsequent angiogenic sprouting and vascular remodelling.[54] Indeed, PI3Kα plays a crucial role in VEGF-A-dependent migration of endothelial cells both *in vitro* and *in vivo*, by activating RhoA. On the contrary, PI3Kα does not appear to be involved in the regulation of endothelial cell viability and survival. Graupera *et al.* have also shown that PI3Kβ is not activated downstream of VEGF-A stimulation, although it regulates *in vitro* microvessel outgrowth induced by GPCR agonists such as SDF-1α, interleukin-8 (IL-8), and S1P. However, S1P-dependent endothelial migration requires both PI3Kβ and PI3Kγ. Although only PI3Kβ mediates Akt phosphorylation, both PI3Kβ and PI3Kγ are instrumental in Rac1 signalling.[55]

The positive effect of PI3K on angiogenesis is counteracted by PTEN. In fact, different groups have characterized PTEN as a negative regulator of angiogenesis both *in vitro*, where it inhibits vascular sprouting and VEGF-A-induced tube formation, and *in vivo*, where PTEN overexpression or administration of PI3K inhibitors block tumour angiogenesis.[56,57] Moreover, mice carrying an endothelial cell-specific mutation of PTEN display enhanced tumourigenesis due to an increased angiogenesis driven by VEGF.[58]

3. Phosphoinositide 3-kinase signalling in vascular smooth muscle cells

VSMCs control the vascular tone through their contractile machinery. Moreover, proliferation and activation of VSMCs represent a primary aspect of vascular remodelling and restenosis. Several lines of evidence have uncovered the function of PI3K/Akt signalling in VSMC biology (*Figure 2*), with PI3Kγ playing a pivotal role in regulating their contractility and proliferation.

The PI3K/Akt axis affects the calcium currents that govern VSMC contraction through coupling membrane receptors to calcium channels.[59] In this respect, Viard *et al.*[60] have shown that the PI3K-induced calcium entry occurs through the phosphorylation of the $Ca_v\beta_{2a}$ subunit of the L-type Ca^{2+} channel on an Akt consensus site, which promotes its translocation to the plasma membrane. Among the main vasoconstrictors which have been shown to activate PI3K/Akt are angiotensin II (AngII) and endothelin-1 (ET-1). AngII turns on the PI3K/Akt pathway and activates L-type Ca^{2+} currents in VSMCs downstream of the AngII type 1

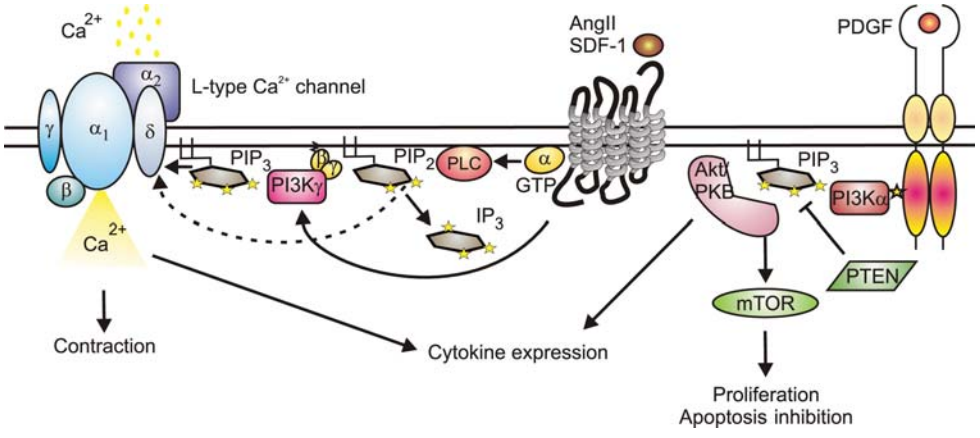

Figure 2 In vascular smooth muscle cells, PI3Kγ signalling regulates contractility and proliferation. By enhancing calcium currents through L-type Ca^{2+} channels, PI3Kγ leads to vasoconstriction (e.g. in response to angiotensin II). Activation of mammalian target of rapamycin kinase, which stimulates cell cycle progression and inhibits apoptosis, is involved in neointimal hyperplasia.

receptor.[61–63] Such response is inhibited by selectively blocking PI3Kγ, thus suggesting a crucial role for this particular PI3K isoform in the regulation of VSMC contractility.[64] In turn, the intracellular infusion of purified PI3Kγ in rat VSMCs mimics the $G_{\beta\gamma}$-induced stimulation of Ca^{2+} channels.[63] *In vivo*, isolated vessels from mice lacking PI3Kγ show reduced contractility in response to AngII, decreased AngII-mediated ROS production, and blunted intracellular Ca^{2+} mobilization. Moreover, knockout mice for PI3Kγ are indeed protected from AngII-induced hypertension and vascular damage.[65] These findings suggest that PI3K inhibition may be considered as a potential anti-hypertensive and vasculo-protective therapy.

With respect to endothelins, pan-PI3K inhibitors can blunt ET-1-dependent calcium currents in rabbit VSMCs and denuded basilar artery preparations.[66,67] In particular, PI3K is activated downstream of ET-receptors and leads to the opening of Ca^{2+}-permeable non-selective cation channel-2 (NSCC-2) and a store-operated Ca^{2+} channel, whereas NSCC-1, another mediator of ET-1-related calcium currents, has been shown to be PI3K-independent. Interestingly, although PI3K is instrumental in the activation of calcium currents, it is not involved in their maintenance, as PI3K inhibitors are effective in preventing and not in blocking ET-1-stimulated calcium entry.[67]

Vascular remodelling is characterized by VSMC activation, leading to matrix deposition, cytokine secretion, and cell proliferation into injured areas. The latter is dominant in the processes of neointimal hyperplasia and restenosis, which occur in arterial segments treated with angioplasty and bare metal stents and may lead to recurrent cardiovascular events. Several lines of evidence have implicated phosphoinositide signalling in such events. It was originally shown that the mitogenic stimulation of VSMCs *in vitro* and in vascular injury *in vivo* converge on the activation of serine/threonine kinase mammalian target of rapamycin (mTOR), leading to the up-regulation of cyclins and the down-regulation of cell cycle inhibitors.[68–72] Such events can be successfully blocked with mTOR inhibitor rapamycin. Further studies have subsequently shown that PI3K/Akt are upstream activators of mTOR in VSMCs. Highly proliferative neointimal VSMCs, similarly to embryonic VSMCs, present high constitutive expression of Akt and mTOR. The pharmacological inhibition of PI3K (with wortmannin or LY-294002), as well as the use of a dominant-negative Akt adenovirus, can suppress VSMC growth similarly to rapamycin.[73] The efficacy of mTOR inhibitors in preventing restenosis has been widely demonstrated with the use of rapamycin-eluting stents in coronary angioplasty.[74,75] In fact, these devices constitute the first widely used and highly effective applications of PI3K/Akt pathway inhibitors in actual clinical practice.

Counteracting PI3K signalling, PTEN reduces VSMC activation. In mice with a VSMC-targeted deletion of PTEN, Akt phosphorylation is increased in vessels, leading to medial hyperplasia, vascular remodelling, and pathological findings suggestive of pulmonary hypertension. Moreover, PTEN deficiency leads to an increased release of SDF-1α, resulting in autocrine stimulation and progenitor cell recruitment. The PI3K/SDF-1/CXCR4 loop can be reversed by hypoxia-inducible factor-1 alpha silencing, suggesting a central role for this transcription factor in the PI3K/Akt pathway in VSMCs.[76] Vice versa, it has been shown that the overexpression of PTEN inhibits growth factor-induced proliferation, migration, and survival of primary rabbit VSMCs.[77] PTEN has the same function also *in vivo*. Adenoviral-mediated overexpression of PTEN in a rat carotid injury model inhibits neointimal hyperplasia through induction of apoptosis and inhibition of cell proliferation.[78] Furthermore, an adenovirus used to overexpress PTEN in the adventitia attenuates cuff-induced neointima formation by reducing cell proliferation, pro-inflammatory cytokines production [C-C chemokine motif ligand 2 (CCL-2), tumour necrosis factor alpha (TNF-α), and interleukin-1 beta (IL-1β)] and increasing adventitial cell apoptosis. Moreover, *in vitro* studies on isolated VSMCs demonstrate that PTEN overexpression inhibits AngII-induced CCL-2 expression through a PI3K-dependent mechanism.[79]

4. Phosphoinositide 3-kinase signalling in atherosclerosis

Atherosclerosis is the leading cause of morbidity, mortality, and disability worldwide, with myocardial infarction and ischaemic stroke representing its major clinical consequences. The atherosclerotic vascular remodelling and pathophysiology involve multiple cell types and a wide array of mediators and cascades. Of note, the PI3K/Akt signalling pathway impinges on several of them. Such functional convergence is challenging for basic and clinical research, but also offers a unique opportunity for pharmacological inhibitors to broadly impact on the biology of atherosclerosis and its complications, bypassing receptor heterogeneity (*Figure 3*).

Inflammation represents a key element of the atherosclerotic process and involves the migration of leucocytes into atherosclerotic lesions and their local contribution through additional chemokine amplification, proteolytic cleavage of the extracellular matrix, and cross-talk with local vascular cells. Indeed, PI3K signalling is a key participant in each of these events. Among the different PI3K isoforms, PI3Kγ is highly expressed in the haematopoietic cell lineage and hence dominates the inflammatory aspects of atherosclerosis. PI3Kγ can be activated by several chemokines (e.g. IL-8, CCL-2/MCP-1, CCL-3/MIP-1α), pro-inflammatory lipids (e.g. platelet-activating factor, leucotriene B4, oxidized LDLs, bacterial components), and vasoactive stimuli (e.g. C5a, AngII), downstream of G_i-coupled receptors.[3,80] Activation of PI3K/Akt signalling has also been shown downstream of other relevant pro-atherogenic stimuli which ligate different receptor types, such as interferon gamma,[81] transforming growth factor beta,[82] and TNF-α.[83]

Although neutrophils mostly migrate into vulnerable plaques, lymphocytes and macrophages are found throughout the atherosclerotic remodelling. Neutrophils and macrophages lacking PI3Kγ present impaired migration towards different chemokine stimuli and defective oxidative burst.[84–87] Moreover, PI3Kγ-selective inhibitors have been shown to exert substantial anti-phlogistic properties *in vivo* in different models of chronic and autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus, as well as in acute conditions such as acute lung injury and sepsis.[88–90] Recently, Fougerat

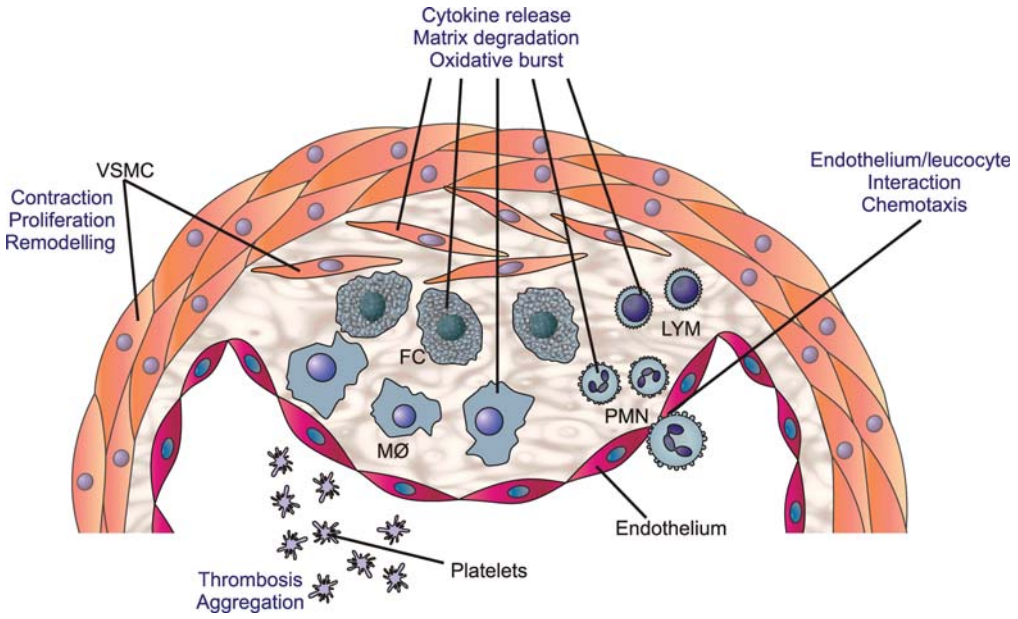

Figure 3 Phosphoinositide 3-kinase/Akt signalling is extensively involved in the biology of atherosclerosis. The specific roles of this pathway in the different cell types are summarized in blue. FC, foam cells; **MØ**, macrophages; LYM, lymphocytes; PMN, polymorphonucleate cells; VSMC, vascular smooth muscle cells.

et al.[91] have tested the efficacy of pharmacological PI3Kγ inhibition (compound AS605240) in murine models of atherosclerosis. In this study, the chronic intraperitoneal administration of AS605240 to apolipoprotein E (ApoE) or LDL receptor (LDLR)-deficient mice significantly reduced the development of early and advanced atherosclerotic lesions. Treatment with PI3Kγ inhibitor was associated with a significant reduction in Akt phosphorylation within plaques, where PI3Kγ mostly co-localizes with macrophage and lymphocyte markers. Interestingly, the anti-atherosclerotic actions of AS605240 were recapitulated in ApoE and in LDLR-deficient mice transplanted with PI3Kγ-deficient bone marrows, suggesting that the atherogenic functions of this PI3Kγ are mostly attributable to its expression in leucocytes. Similar findings have been previously reported in PI3Kγ-ApoE double knockout mice, which exhibit reduced lesion size compared with single ApoE-knockout controls.[80] In these animals, the absence of PI3Kγ was sufficient to suppress, within atherosclerotic plaques, the phosphorylation of several downstream PI3-kinase/Akt targets, including GSK3, p70S6kinase, S6 ribosomal protein, and PKCθ, confirming the non-redundancy of PI3Kγ functions in this process. Contrasting data has been provided for the entity of macrophage infiltration upon the absence or blockade of PI3Kγ. Although Fougerat have reported reduced macrophage content in AS605240-treated mice, overall macrophage density was apparently unchanged in the study by Chang *et al.*[80] Such findings suggest that the potential anti-atherogenic properties of PI3K inhibitors may depend on several factors and not only on the limitation of leucocyte migration. An important vascular effector of PI3K is Akt1, which plays a determinant role in atheroprotection. In double ApoE-Akt1 knockout mice, atherosclerotic lesions in the aorta and coronary vessels are more severe than in ApoE-knockout controls. Interestingly, no modification of the atherosclerotic load is observed in ApoE-knockout mice transplanted with the bone marrow of donor double ApoE-Akt1 knockout mice, hence suggesting that the anti-atherogenic roles of Akt1 derive from vascular cells. Loss of Akt1 in the vessel wall is indeed associated with increased inflammatory signalling and reduced eNOS phosphorylation.[92] Thus, PI3Kγ/Akt1 should be regarded as a fundamental molecular axis for the pathobiology of atherosclerosis.

5. Phosphoinositide 3-kinase signalling in platelets

Inappropriate platelet activation and thrombus formation represent pathophysiological milestones of the atherosclerotic disease. In fact, acute complications of atherosclerotic plaques (e.g. plaque rupture or fissuration) trigger platelet aggregation and hence initiate dramatic clinical events such as myocardial infarction and stroke. Several factors regulate platelet adhesion and aggregation, including the functional state of cellular enzymes, membrane receptors, and glycoproteins. Among the main pro-thrombotic platelet receptors are ADP ($P2Y_1$, $P2Y_{12}$), thromboxane A_2, and thrombin (protease-activated receptor-1 and -4) receptors, whereas essential proteins securing adhesion and aggregation to substrates and other platelets are glycoproteins GPIb/V/IX, GPVI, and integrin GPIIb/IIIa (also known as $\alpha_{IIb}\beta_3$). Interestingly, different lines of evidence have implicated PI3K/Akt in such signalling pathways (*Figure 4*). On these grounds, the pharmacological modulation of PI3K/Akt has emerged as a novel potential therapeutic approach to anti-thrombotic therapy.

Recent work has established that the molecular mechanisms underlying platelet functions are profoundly affected by local haemorheological conditions. In arterial thrombosis, the first phase of thrombus formation is initiated by the contact of platelets with subendothelial molecules, including vWF, fibrinogen, and collagen. In such shear stress situations, platelets first roll on and tether to the subendothelial layer through the interaction of glycoprotein GPIb/V/IX with immobilized matrix-bound vWF. This event starts a signalling cascade culminating in integrin GPIIb/IIIa

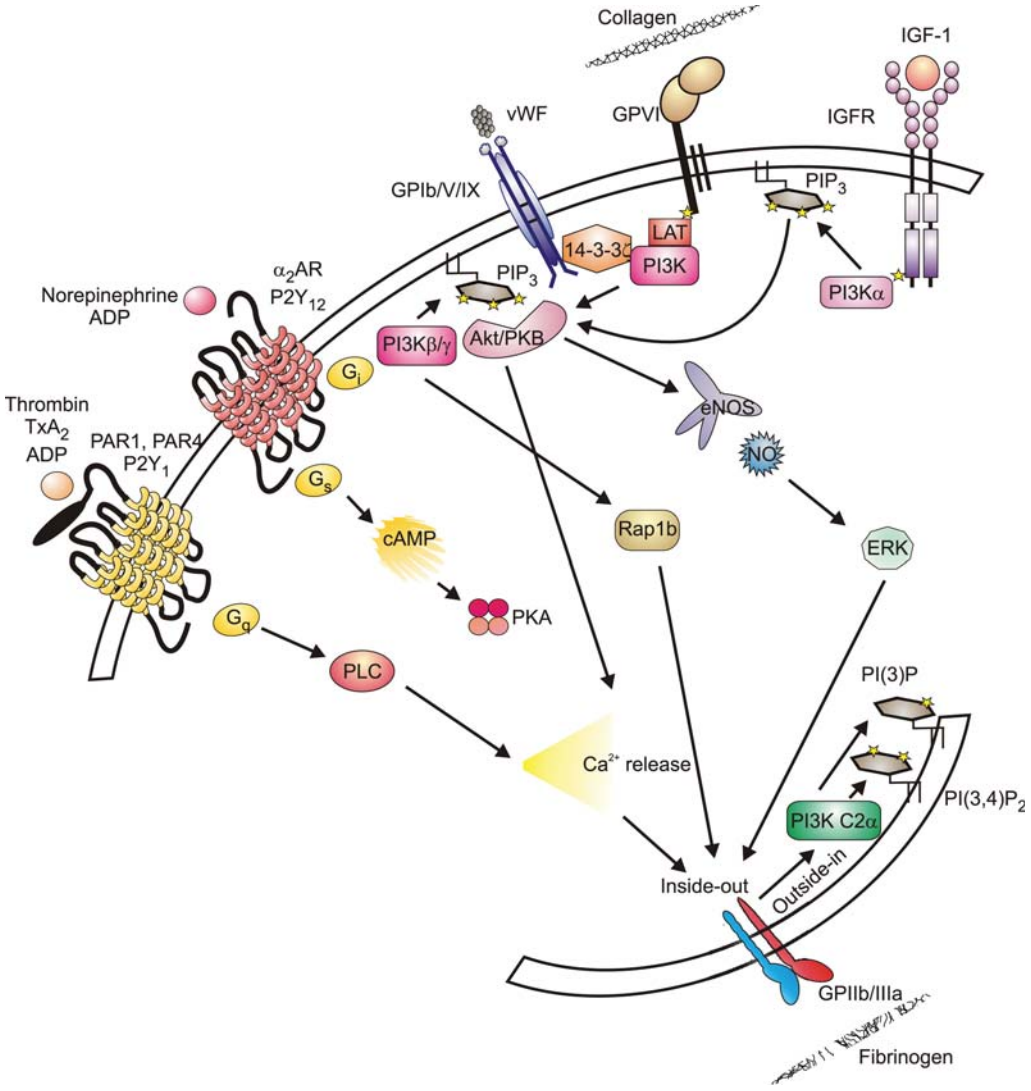

Figure 4 In platelets, phosphoinositide 3-kinase signalling is widely involved in the regulation of adhesion and aggregation. Phosphoinositide 3-kinases are activated downstream of several membrane proteins, including G-protein-coupled receptors (e.g. $P2Y_{12}$), tyrosine kinases (e.g. IGFR), GPIIb/IIIa, GPIb/V/IV, and GPVI. In turn, phosphoinositide 3-kinases/Akt promote thrombosis through enhanced calcium release and GPIIb/IIIa activation.

activation (a process indicated as inside-out signalling), which assures firmer binding to vWF and fibrinogen.[93] Jackson *et al.*[94] originally reported that the exposure of platelets to vWF causes the association to cytoskeleton and the functional activation of PI3K. Upon binding to vWF, GPIb/V/IX C-terminus first independently interacts with PI3K and signalling protein 14-3-3ζ.[95,96] Such interaction is shear–stress-specific, since inhibition of PI3K with wortmannin or LY294002 only marginally affects basal platelet function. In the presence of shear stress, PI3K inhibitors dramatically reduce platelet adhesion and spreading by blunting calcium mobilization and GPIIb/IIIa activation, resulting in impaired thrombus growth.[97] Though different class I PI3K isoforms are expressed in platelets (PI3Kα, β, γ), PI3Kβ appears to be the one critically involved in this pathway, regulating the formation and stability of integrin GPIIb/IIIa bonds.[98,99] Notably, an isoform-selective PI3Kβ inhibitor (TGX-221) has been shown to impair thrombus formation also *in vivo* without prolonging bleeding time. Furthermore, vWF-GPIb/V/IX-induced platelet aggregation and adhesion are impaired in Akt1 and Akt2-deficient platelets, as well as in platelets treated with an Akt inhibitor (SH-6), suggesting sequential class I PI3K and Akt activation upon inside-out signalling.[100] Downstream, Akt has been suggested to act in turn through the activation of eNOS, ultimately triggering a cGMP-p38-Erk signalling cascade.[101,102] This is a relatively unusual situation, since stimuli triggering PI3K commonly activate the MAPK pathway in parallel. Other examples of such condition implicate the involvement of PI3Kγ in Erk activation through the direct phosphorylation of Mek1, but the precise role of this process in cardiovascular functions is not known.[103]

Exposure of platelets to fibrinogen implies direct GPIIb/IIIa activation and triggers a so-called outside-in signalling pathway, which stabilizes the cytoskeleton and contributes to shape change. Therefore, PI3Ks are involved in both inside-out and outside-in platelet signalling. However, although fibrinogen-GPIIb/IIIa interaction is followed by PtdIns(3,4)P_2 production, PtdIns(3,4,5)P_3 does not appear to accumulate in this condition.[104–106] This finding indicates that outside-in signalling may be associated with class II (possibly isoform C2α) and not with class I PI3K activation. Accordingly, outside-in integrin signalling does not appear to implicate Akt. In fact, Akt deficiency or inhibition does not impair

fibrinogen-induced platelet aggregation, whereas pan-PI3K inhibitors blunt both outside-in and inside-out signalling.[100]

Finally, subendothelial collagen is bound by glycoprotein GPVI, which then complexes and cross-links to the FcRγ chain. The main consequence of GPVI-FcRγ-chain signalling is PLCγ activation and calcium release. However, such events also involve the accumulation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 and are attenuated by pan-PI3K inhibitors. These findings prompt to the participation of PI3K even in collagen-triggered platelet aggregation.[107] PI3K appears to interact with tyrosine-phosphorylated FcRγ, as well as with the adapter protein linker for activator of T cells through the SH2 domains of the PI3K regulatory subunit, p85α. Accordingly, collagen-induced platelet aggregation is specifically impaired in p85α-deficient mice.[108]

Following initiation, thrombus formation proceeds with a propagation phase, characterized by the release of several autocrine/paracrine mediators from dense granules, including ADP. While ADP receptor $P2Y_1$ signals through G_s-PLC, $P2Y_{12}$ (as α_{2a}-adrenergic receptor) is a G_i-coupled receptor. G_i signalling involves both the inhibition of adenyl cyclase/PKA and the activation of class I PI3Ks. In turn, PI3Ks signal towards GPIIb/IIIa, following at least two downstream pathways. First, class I PI3Ks activate GTPase Rap1b, which in turn functions as an activator and stabilizer of GPIIb/IIIa.[109,110] Secondly, PI3K signalling strengthens and prolongs G_s-dependent signalling. In thrombin-stimulated platelets, ADP-$P2Y_{12}$-PI3K enhance the long-term calcium mobilization induced by G_s-coupled thrombin receptor. Interestingly, PI3Kβ, and not the prototypical GPCR-activated PI3Kγ, appears to be the dominant isoform mediating this effect, as shown with the use of PI3Kβ-selective inhibitor TGX221 and in PI3Kγ-defective platelets.[111] Moreover, PI3Kβ has been identified as the dominant PI3K isoform responsible for G_i-dependent integrin $\alpha_{IIb}\beta_3$ activation following ADP stimulation, as demonstrated by the loss of ADP-induced aggregation in the presence of PI3Kβ-selective inhibitor TGX221. However, also PI3Kγ appears to be instrumental for integrin $\alpha_{IIb}\beta_3$ activation downstream of $P2Y_{12}$, cooperating with PI3Kβ. *In vivo*, arterial thrombus formation is indeed impaired in PI3Kγ-deficient mice and completely abolished with the addition of a PI3Kβ inhibitor.[112] Furthermore, it has been shown that PI3Kγ-deficient mice exhibit reduced susceptibility to venous thromboembolism, though maintaining a normal bleeding time.[98] Interestingly, platelet PI3Kγ appears to exert its effects through kinase-independent mechanisms. In fact, upon PI3Kβ inhibition, no G_i-induced Akt phosphorylation is detected and PI3Kγ-selective inhibitor AS252424 does not affect thrombin-induced calcium currents.[111,113] It is noteworthy that a kinase-independent function of PI3K is quite unique and has been identified to date in platelets, cardiomyocytes, and EPCs.[38,114] Although in cardiomyocytes the kinase-independent function of PI3Kγ involves the regulation of phosphodiesterase 3B activity and cellular cAMP levels, it is unclear if a similar pathway is also operational in platelets.

Another autocrine/paracrine platelet mediator is IGF-1, which acts as a pro-aggregant adjuvant through binding to surface receptors. Recent work has unveiled a specific role for PI3Kα in this pathway. Interestingly, the corroborating effect of IGF-1 on platelet aggregation is blunted by pan-PI3K inhibitor wortmannin as well as by a PI3Kα-selective inhibitor (PIK-75). Therefore, PI3Kα selectively contributes to Akt phosphorylation downstream of IGF-1 stimulation in the presence of G_i-dependent signalling.[115]

6. Summary and perspectives

The PI3K/Akt pathway participates in numerous cellular functions underlying vascular physiology and disease. In the endothelium, the PI3K/Akt signalling mostly acts as a positive regulator of eNOS and angiogenesis. Moreover, recent findings have pinpointed its role in promoting EPC viability, number, and function. In VSMCs, the PI3K/PTEN/Akt pathway modulates contractility and, mostly through mTOR, it orchestrates the cellular responses to mitogenic stimulation. Accordingly, PI3K and mTOR inhibition protects from restenosis and neointimal formation. In platelets, PI3Kγ and PI3Kβ play pivotal roles in aggregation and thrombosis. Finally, PI3Kγ is a key positive regulator of inflammatory signalling in macrophages within atherosclerotic remodelling. Beyond providing fundamental knowledge, this bulk of evidence has suggested that the pharmacological targeting of the PI3K/Akt pathway is appealing and potentially amenable for therapeutic purposes in atherosclerotic vascular disease and its complications. The development of isoform-selective PI3K inhibitors has especially fostered this perspective and awaits further translational research and clinical trial.

Conflict of interest: E.H. also operates as a consultant for Merck Serono and Cellzome.

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