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Visions & Reflections

Type I phosphoinositide 3-kinases: potential antithrombotic targets?

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Abstract. Arterial thrombosis is the single most common cause of death and disability in industrialized societies and is the primary pathogenic mechanism underlying acute myocardial infarction and ischemic stroke. Platelets play a central role in this process, and as a consequence, a great deal of effort has gone into identifying the mechanisms regulating the adhesive function of platelets. Platelet adhesion is controlled by intracellular signaling pathways, with growing evidence for a major role for phosphoinositide 3-kinases (PI3Ks) in this process. Platelets express all type I PI3K isoforms, including p110α, p110β, p110δ and p110γ, with recent evidence suggesting important roles for p110γ and p110β in regulating distinct phases of the platelet activation process. Deficiency of p110γ or inhibition of p110β produces a marked defect in arterial thrombosis without a corresponding increase in bleeding time, raising the possibility that inhibition of one or more PI3K isoforms may represent an effective antithrombotic approach.

Keywords: Haemostasis and thrombosis, PI 3-kinase isoforms, platelet activation, signal transduction.

The phosphoinositide 3-kinases (PI3Ks) represent a family of structurally and functionally distinct lipid kinases which phosphorylate the 3-hydroxyl position of membrane phosphoinositides, generating at least three distinct lipid products, including PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3. They are subclassified into three distinct groups based on their substrate specificity, primary sequences and mode of regulation [1–3]. The type I enzymes are the most intensely studied group due to their role in stimulus-response coupling downstream of a wide variety of cell surface receptors. There are four type I isoforms, p110α, p110β, p110δ and p110γ, all of which are expressed in platelets [4–6]; however, delineation of the roles of each of these isoforms in platelet function has been slow to develop due to the lack of available isoform selective inhibitors and, as is the case for p110α and p110β, due to the lack of viable mouse models [7–9].

A central role for PI3Ks in platelets

Central to the haemostatic function of platelets is their ability to undergo a series of morphological and biochemical responses linked to primary adhesion, shape change, aggregation, granule release and the exposure of a procoagulant surface [10, 11]. Based on studies using the PI3-kinase pharmacological inhibitors, LY294002 and wortmannin [12], there is evidence for a role for PI3Ks in the regulation of almost all these functional responses (Table 1) [13–24]. This, combined with the demonstration that all physiological stimuli can induce PI3K activation [17, 25–30], has suggested a fundamentally important role for these enzymes in platelet function.

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Table 1. Investigation of type I PI3K, platelet function.

Strategy	Limitations; effects on other PI3Ks	Effects on haemostasis and/or thrombosis in vivo	Abnormalities in platelet signaling and function
Pan-PI3K inhibitors (wortmannin, LY294002)	both inhibit all type I PI3Ks; wortmannin inhibits type II PI3K-C2β at nM concentrations [12]	protection against occlusive thrombus formation (Folt's type model) [36]	– impaired aggregation in response to ADP, shear and threshold concentrations of other agonists – impaired FGN binding in response to ADP – impaired spreading under static conditions – reduced stable adhesion, calcium flux and aggregate formation under shear conditions – impaired $P2Y_{12}/G_i$ mediated signaling, including Rap1b activation [13-24]
p85α knockout [4, 31,32]	p110α/β/δ absent or reduced (5% residual kinase activity) [4]	no overt bleeding disorders; normal tail bleeding time [4]	– normal aggregation in response to ADP, TxA2, thrombin, PMA, A23187, botrocetin – impaired aggregation, p-selectin expression and FGN binding in response to CRP – reduced lamellipodia formation on collagen and CRP – partial abrogation of Btk, Tec, Akt and PLCγ2 phosphorylation in response to CRP/collagen [4]
p110β-selective inhibitors (TGX221) [36]	100- and 1000-fold selective over δ, and α/γ, respectively [36]	protection against occlusive thrombus formation (Folt's and electrolytic type models); normal tail bleeding time [36]	– impaired aggregation in response to ADP and threshold concentrations of all other agonists – impaired FGN binding in response to ADP – normal calcium flux and shape change in response to ADP – impaired $G_i/P2Y_{12}$-mediated signaling, including activation of Rap1b – reduced stable adhesion and calcium flux under shear conditions [36]
p110δ knockout [34]	normal expression of p110α, β [34, 35], small reduction in p85/55/50α [34]	no overt bleeding disorders [34]	– normal aggregation in response to ADP, TxA2, thrombin, PMA, A23187, botrocetin – impaired p-selectin expression and FGN binding in response to CRP – reduced lamellipodia formation on collagen and CRP (static conditions), but normal stationary adhesion – slight decrease in Btk and PLCγ2 phosphorylation in response to CRP [35] – normal adhesion to FGN and calcium flux under shear conditions [36]
Kinase dead p110δ [33]	normal protein expression of p110α,β,δ, as well as p85/55/50; normal p110α,β kinase activity [22]		– mainly consistent with findings for p110δ-deficient platelets [35]
p110δ-selective inhibitors D-010/IC87114	40-fold and >150-fold selective over p110γ and p110α/β, respectively [40]	no protection against occlusive thrombus formation in Folt's type Model [36]	– no overt defects in aggregation (thrombin, ADP) – no defects in FGN binding in response to ADP
p110γ knockout [37]	normal protein expression of p110α,β and δ [37,38]	protection against ADP-induced thromboembolism [38]; unstable thrombi in $FeCl_3$-induced carotid injury [39]; no protection against occlusive thrombus formation in Folt's type injury model [36]; normal tail bleeding time [38]	– impaired aggregation in response to ADP [38,39] – normal aggregation [38] and actin filament assembly [39] in response to other agonists (thrombin, collagen) – normal calcium flux and adenylyl cyclase inhibition in response to ADP [38] – reduced Akt phosphorylation and FGN-binding in response to ADP, and reduced spreading on FGN [38]

Thus, it is surprising that despite intense investigation it has yet to be clearly established whether PI3K signaling processes are central to the normal haemostatic function of platelets as there are currently no descriptions of bleeding diatheses in humans or mice attributable to defects in PI3K signaling.

Importance of type I PI3K in platelets

Type Ia PI3Ks

Most of the information on the role of PI3K isoforms in platelets has been derived from studies on mice that have undergone genetic manipulation of specific PI3K regulatory or catalytic subunits (Table 1). Deletion of the p85 regulatory subunit has no significant affect on the expression of other type Ia regulatory subunits; however, it leads to a major reduction in the levels of the p110α catalytic subunit, along with greatly reduced p110β and δ levels [4, 31, 32], providing a useful model system to investigate the role of type Ia PI3Ks. Significantly, these platelets retain a normal platelet aggregation response to a wide range of physiological agonists, including ADP, TxA2 (U46619), thrombin and vWf/botrocetin [4]. However, platelet aggregation, P-selectin expression and spreading in response to collagen and collagen-related peptide (CRP) was significantly perturbed in p85α null platelets [4], suggesting a potentially important role for these enzymes in GPVI signaling (Fig. 1). Whether the normal aggregation response of p85α null platelets to soluble agonists reflects residual signaling by type Ia isoforms or is due to partial compensation by p110γ remains to be established.

Of the type Ia catalytic subunits, only deletion of p110δ has allowed the generation of viable progeny [33, 34], and analysis of thymocytes from these mice has revealed normal levels of p110α and p110β [34]. Functional studies on p110$\delta^{-/-}$ platelets have revealed a minor role for this isoform in platelet function, consistent with the low level of p110δ expression. p110$\delta^{-/-}$ platelets aggregate normally in response to ADP, thrombin and high concentrations of CRP [35], adhere and spread normally on an immobilized collagen matrix, as well as exhibit normal shear-dependent adhesion and aggregate formation on collagen, fibrinogen or vWf/fibrinogen matrices [35]. Platelet aggregation in response to threshold concentra-

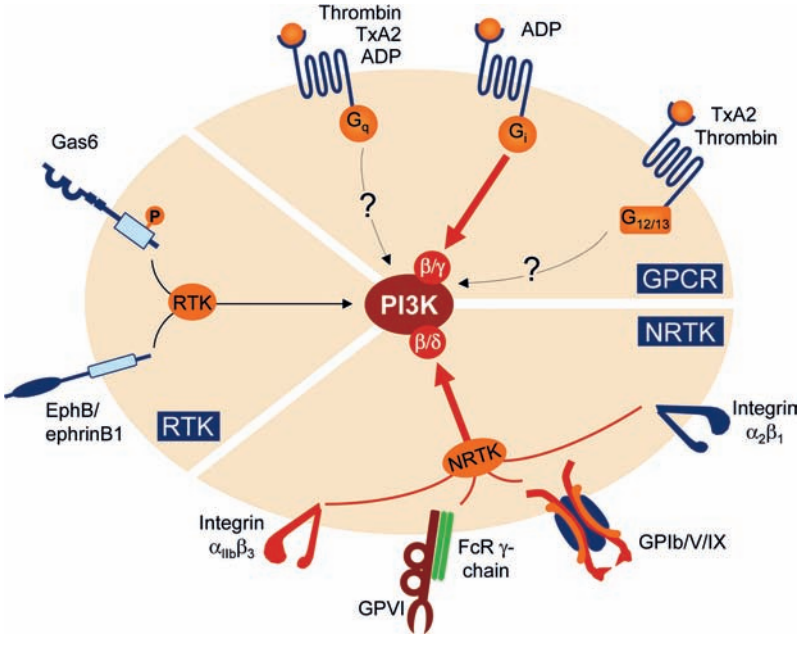

Figure 1. Ligand/receptor interactions utilizing type I PI3K signaling processes in platelets. Platelets express a range of receptors on their surface membrane, which are recognized by a variety of soluble or immobilized ligands. The receptor/ligand interactions reported to utilize PI3K to modulate platelet function are depicted in this figure. For simplicity we have divided these receptors into G-protein (GPCR)-, receptor tyrosine kinase (RTK)- and non-receptor tyrosine kinase (NRTK)-coupled receptors. GPCRs for the soluble agonists ADP, TxA2 and thrombin are further divided into the separate G-protein subtypes G_q, G_i and $G_{12/13}$. Adhesion receptors which utilize NRTKs to transduce signals include integrins ($\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$), the leucine-rich glycoproteins (GPIb/V/IX) and ITAM-containing receptors (such as GPVI). RTKs for Gas6 and ephrinB1 (EphB) are also depicted, as recent studies have suggested a potentially important role for PI3K signaling processes downstream of these receptors. The relationship between GPCRs, NRTKs or RTKs and the activation of PI3K isoforms is highlighted by the different arrows. The red bolded arrows demonstrate receptors in which PI3K has been demonstrated to play an important signaling role. Currently, there is evidence that p110β is involved in signaling processes downstream of both NRTK and GPCRs, p110δ in NRTK signaling and p110γ in GPCR signaling. The PI3K isoforms responsible for signaling downstream from the Gas6 RTKs as well as EphB remain to be defined, as depicted by the thin black arrows. Broken arrows with question marks downstream from G_q and $G_{12/13}$ indicate that there is insufficient evidence to support a major signaling role for PI3K.

tions of CRP was slightly reduced in these mice, suggesting a minor role for p110δ in GPVI signaling (Fig. 1).

To date, there have been no reports of viable mice expressing mutant or deletion forms of p110α or p110β, suggesting an indispensable role for these enzymes in normal mouse development [8, 9]. To investigate the role of p110β in platelets, we have recently developed isoform-selective inhibitors that have approximately 100–1000 fold selectivity for p110β over p110α and p110γ, and 20–100-fold selectivity over p110δ [36]. In combination with mice lacking p110δ and p110γ, these inhibitors are effective at delineating the role of p110β in platelets. Platelets pretreated with p110β inhibitors demonstrate normal granule release in response to a wide range of physiological agonists and exhibit normal integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation in response to stimulation by high-dose thrombin, TxA2 analogue (U46619) and collagen [36]. The major effect of inhibiting p110β is the inability of platelets to sustain integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation in response to ADP and threshold doses of other soluble agonists. Moreover, inhibiting p110β prevents the development of stable integrin $\alpha_{IIb}\beta_3$ adhesion contacts under high shear, leading to defective shear-induced platelet aggregation [36]. Taken together, these studies define an important role for p110β in sustaining integrin $\alpha_{IIb}\beta_3$ activation (Fig. 1) necessary for stable platelet adhesion and aggregation.

Type Ib PI3Ks

Mice lacking p110γ are fertile, developmentally normal and demonstrate normal expression of other PI3K catalytic subunits [37]. Analysis of platelets from these mice demonstrate normal ATP secretion, F-actin assembly and normal aggregation profiles in response to thrombin and high-dose collagen [38, 39]. p110$\gamma^{-/-}$ platelets do, however, have an impaired aggregation response to ADP and threshold concentrations of collagen [38, 39] and a reduced spreading response on immobilized fibrinogen [39]. These defects may in part be explained by impaired ADP-induced integrin $\alpha_{IIb}\beta_3$ activation. Detailed biochemical analysis of p110$\gamma^{-/-}$ platelets have revealed a defect in signaling through the ADP purinergic receptor $P2Y_{12}$, leading to impaired Akt activation [38]. Interestingly, a defect in $P2Y_{12}$ signaling has also been observed in human and mouse platelets treated with isoform-selective p110β inhibitors [36], suggesting a possible role for multiple PI3K isoforms in this pathway (Fig. 1).

Importance of PI3K signaling for arterial thrombosis

One of the most significant observations from the study of p110γ null mice and animals treated with isoform-selective p110β inhibitors is the marked defect in arterial thrombus formation without a concomitant increase in bleeding time. For example, p110γ null mice are protected against death from acute ADP-induced thromboembolism [38], and form unstable arterial thrombi in a chemical-induced carotid artery thrombosis model [39]. However, the time to haemostatic plug formation following tail transection is normal [38]. Similarly, pretreating rats and rabbits with p110β inhibitors abolishes occlusive thrombus formation in a 'Folts-type' stenosis-injury carotid thrombosis model [36] and was more effective than aspirin at preventing arterial occlusion following electrolytic injury [36]. Even at concentrations well above those required for an antithrombotic effect, p110β inhibitors had minimal effect on bleeding time even when administered in combination with other antithrombotic agents [36]. These findings have raised the possibility that selective targeting of either p110γ or p110β may represent an effective antithrombotic strategy that is devoid of troublesome bleeding side-effects.

Isoform-selective p110γ inhibitors have recently been developed by a number of pharmaceutical companies [40, 41] and have been demonstrated to have potent anti-inflammatory activities in a number of different animal models [42, 43]. However, there are currently no reports on the antithrombotic potential of these inhibitors. Nonetheless, given that deficiency of p110γ in mice is very well tolerated, with no obvious fertility or developmental defects, there is considerable enthusiasm that such compounds may represent safe, effective therapeutic agents. The tolerance of p110β inhibitors remains uncertain, with concern over the ubiquitous expression of this isoform and the demonstrated embryonic lethality of p110β null mice. Whether partial inhibition of the kinase by pharmacological approaches will be more effectively tolerated than complete deficiency of the kinase remains to be established. From an antithrombotic perspective, short-duration therapies for the management of acute coronary syndromes and ischemic stroke are important, and the possibility of using PI3K inhibitors in combination with other antithrombotic agents without increasing bleeding risk, particularly intracerebral hemorrhage, is highly attractive.

Reflections

Significant progress has been made over the last few years in defining the role of individual type I PI3K isoforms in platelets and the arterial thrombotic process. In contrast to other haemopoietic cells [44], p110δ appears to play a relatively minor role in platelets with the mild defect in GPVI signaling (Fig. 1) not associated with significant abnormalities in the haemostatic function of platelets [35, 36]. In contrast, p110γ, which is also highly expressed in haemopoietic cells, appears to play a

significant role in promoting ADP-induced platelet activation necessary for thrombus formation in several distinct experimental models [38, 39]. Recent studies have demonstrated that p110γ can potentially signal through both catalytic and non-catalytic mechanisms [45], and it remains to be established which of these signaling mechanisms is relevant to the defect in platelet function. This may be potentially important, as pharmacological inhibition of the catalytic function of p110γ may not produce the same level of antithrombotic protection as targeted deletion of p110γ. Furthermore, it remains to be established whether p110γ or p110β is the dominant isoform acting downstream of the G_i-coupled $P2Y_{12}$ receptor (Fig. 1). There is evidence in neutrophils that p110γ is the predominant isoform responsible for G_i-dependent $PI(3,4,5)P_3$ formation necessary for superoxide generation [44], whereas in non-haemopoietic cells, it has been proposed that p110β is the dominant isoform transducing G_i signals [46].

Of the two ubiquitously expressed type I PI3K isoforms, p110α and p110β, there is currently minimal information on the former, whereas the latter appears to play an important role in regulating the adhesive function of integrin $\alpha_{IIb}\beta_3$ [36]. This receptor plays a central role in the thrombotic process, and it is therefore not surprising that inhibition of p110β leads to a significant defect in arterial thrombosis in experimental models [36]. PI3Ks are well known to regulate the adhesive function of integrins in a variety of cell types [13, 15, 47–49], and given the ubiquitous expression of p110β, it is possible that p110β plays a broader role in regulating cell adhesion processes.

While progress has been made, a number of fundamental aspects of PI3K signaling have yet to be clearly defined in platelets. For example, while there is good evidence for an important role for PI3K isoforms in tyrosine kinase-linked signaling pathways linked to adhesion receptors and immunoreceptor tyrosine-based activation motif (ITAM)-bearing receptors, there is still limited information on whether these enzymes play an important role in G_q and G_{13}-signaling processes, linked to the initiation of integrin $\alpha_{IIb}\beta_3$ activation. Moreover, it is still not clear in the pathways utilizing PI3K whether a single isoform or multiple isoforms are involved and how these signaling processes co-ordinate to regulate specific functional responses. There is also limited understanding of how individual PI3K isoforms regulate the various phases of thrombus formation and consolidation. Several of the key receptors utilizing PI3K signaling processes, including GPVI, integrin $\alpha_{IIb}\beta_3$ and $P2Y_{12}$, regulate numerous aspects of platelet function, including procoagulant and proinflammatory activities. Thus it is conceivable that PI3K inhibitors may have significant antithrombotic actions beyond regulating the adhesive function of platelets.

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