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The role of inositol polyphosphate 4-phosphatase 1 in platelet function using a weeble mouse model

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Introduction

Platelet activation plays an important role in the development and course of cardiovascular disease. It is triggered by the interaction of subendothelial matrix-bound and/or soluble agonists with platelet surface receptors causing a series of morphological and biochemical changes leading to the recruitment of additional platelets and formation of stable platelet aggregates. In addition to events causing initial activation and recruitment of platelets, signaling continues post-aggregation that promotes stability of the thrombus (Brass *et al.*, 2004). Platelet levels of phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P₂) increase dramatically in response to agonist stimulation in an aggregation-dependent manner (Kucera *et al.*, 1990; Nolan *et al.*, 1990; Sultan *et al.*, 1990). Furthermore the increase in platelet PtdIns(3,4)P₂ occurs late in platelet aggregation and correlates with the irreversible phase of platelet aggregation (Sorisky *et al.*, 1992; Sultan *et al.*, 1991; Trumel *et al.*, 1999), suggesting that PtdIns(3,4)P₂ mediates the stabilization of platelet aggregates. However little is known about the regulation of PtdIns(3,4)P₂ in platelets. PtdIns(3,4)P₂ can be formed by three different routes 1) by direct phosphorylation of PtdIns(4)P by phosphatidylinositol 3-kinase (EC2.7.1.153) (PI 3-K) 2) by PI 3-K using PtdIns(4,5)P₂ as a substrate followed by dephosphorylation by a 5-phosphatase (EC3.1.3.56) and, 3) by the action of type I PtdIns(4)P phosphate kinase (EC2.7.1.68) using PI 3-K as substrate (Zhang *et al.*, 1998), shown diagrammatically in figure 1. It is well documented that different PI 3-K isoforms play important roles in both early and later stages of platelet aggregation (Jackson *et al.*, 2006). It has been shown that that pharmacologic inhibition of PI 3-K prevents agonist-induced formation of PtdIns(3,4)P₂ (Kovacovics *et al.*, 1995; Schoenwaelder *et al.*, 2007). Furthermore addition of PI 3-K inhibitors after the onset of platelet aggregation induces a decline in PtdIns(3,4)P₂ and disaggregation of platelets, supporting a role for PtdIns(3,4)P₂ in the stabilization of aggregates. However, platelet agonist-dependent activation of PI 3-K mediates an increase in both PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ making approaches using pharmacologic inhibition or genetic disruption of PI 3-K unsuitable to distinguish the contributions of the individual 3-phosphorylated phosphoinositides to platelet signaling. The levels of inositol lipids in cells and their distribution in discrete cellular compartments are regulated by the balance of the enzymatic activities of kinases and phosphatases.

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The major route of PtdIns(3,4)P₂ hydrolysis is the removal of the D4 phosphate by the enzymes cloned and characterized in our lab (Norris *et al.*, 1997; Norris *et al.*, 1995), inositol polyphosphate 4-phosphatase type I (EC3.1.3.40) and type II (EC3.1.3.66) that are magnesium-independent phosphatases. Over their entire sequence type I and type II 4-phosphatases are 37% identical (Norris *et al.*, 1997). The active site region is more highly conserved, and contains a consensus sequence found in other magnesium-independent phosphatases (Zhang *et al.*, 1994). The consensus sequence CX₅ RT/S, is conserved throughout 4-ptases from *C. elegans* to humans as shown in figure 2. These enzymes do not catalyze the hydrolysis of lipids other than PtdIns(3,4)P₂ and therefore provide unique means for the study of this lipid in platelet activation. We have shown that 4-ptase I forms a complex with PI 3-K in platelets which localizes the complex to sites of PtdIns(3,4)P₂ production (Munday *et al.*, 1999).

We postulate that PtdIns(3,4)P₂ is important for platelet function and will study this using a mouse model. We previously showed that an antibody that reacts with 4-phosphatases immunoprecipitates all of the PtdIns(3,4)P₂ hydrolyzing activity from human platelets (Munday *et al.*, 1999). An early indication that PtdIns(3,4)P₂ was important for platelet function was the work of Norris (Norris *et al.*, 1997). It was shown that calpain caused degradation of recombinant 4-phosphatase I *in vitro* thereby inactivating it. It was also shown that activation of human platelets with either calcium ionophore or thrombin led to proteolysis of endogenous platelet 4-phosphatase I. If calpeptin, a cell-permeable inhibitor of calpain, was included in these experiments no proteolysis was seen. The levels of PtdIns(3,4)P₂ in platelets were lower when calpeptin was included, indicating that 4-phosphatase I was important for controlling the levels of PtdIns(3,4)P₂ during platelet activation.

A naturally occurring mutation in type I 4-phosphatase is a single nucleotide deletion which is found in the weeble mouse. These animals suffer from severe neurodegeneration and die within the first weeks of life. Therefore such mutant mice cannot be used to study platelet function. We circumvented this problem by creating chimeric mice by bone marrow transplantation of weeble fetal liver cells into lethally irradiated wild type mice. These mice lack 4-phosphatase in bone marrow derived cells including platelets. The mice are viable, but lack platelet 4-phosphatase I.

Results and discussion

To study the role of 4-phosphatase I in the regulation of PtdIns(3,4)P₂ in platelets we have obtained mice heterozygous for the weeble mutation from Arne Nystuen, University of Nebraska. The mutation arose spontaneously in the Jackson laboratory mouse colony on a C57Bl/6J background and was identified as a single nucleotide deletion (Δ744G) that led to a frame shift and premature stop codon (Nystuen *et al.*, 2001) (figure 3). Subsequently it was backcrossed 10 generations onto a Balb/cByJ background. The mice homozygous for the mutation lack 4-phosphatase I mRNA and protein and are therefore a good model for studying the functions of 4-phosphatase I. Homozygous mice die in the first weeks of life due to severe neurodegeneration and therefore cannot be used to study platelet function. Initially we measured platelet counts in 3 day old weeble mice and found that they were normal. Thus the animals do not have a defect in thrombocytopoiesis. We then generated radiation chimeras by transplanting weeble fetal liver cells into lethally irradiated wild type mice using the protocol of Hirbe (Hirbe *et al.*, 2007), shown in figure 4. Platelet counts were obtained 4 weeks after transplantation and were found to be normal with wild type mice having 766,000 and weeble mice 718,000 (p=0.68). Platelet lysates from weeble chimeras contained no detectable 4-phosphatase I.

We have initiated studies to test the possibility that weeble chimeric mice have a propensity for thrombosis using a carotid artery injury model (He *et al.*, 2002). In this method a segment of mouse carotid artery is isolated and an ultrasonic flow probe is placed distal to the isolated segment in order to measure blood flow. The isolated artery is irradiated with laser light and Rose bengal is injected into the mouse tail vein. The dye is induced to generate free radicals by the laser light which damage the carotid artery causing it to thrombose. The time to cessation of blood flow is measured indicating complete carotid occlusion. In a preliminary experiment the wild type carotid artery thrombosed after 45 minutes (figure 5a) while that from the weeble chimera thrombosed at 22 minutes (figure 5b), a dramatic difference in this model. In future experiments we will measure parameters of *in vitro* platelet function including platelet aggregation and secretion responses to platelet agonists such as ADP, collagen, and thrombin. We will also measure bleeding time, clot retraction (Leon *et al.* 2007), and rates of thrombin generation after activation of platelets *in vitro*. In additional studies using the carotid artery injury model we will determine whether the increased propensity to thrombosis is abrogated by treatment of mice with aspirin to inhibit thromboxane formation (Roth *et al.*, 1975) or with an ADP P2Y₁₂ receptor antagonist (Daniel *et al.*, 1998; Jin *et al.*, 1998). Downstream targets of PtdIns(3,4)P₂ will also be studied including Akt activation (Kroner *et al.*, 2000) and its phosphorylation and Rap1b activation (Woulfe *et al.*, 2002).

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Figure 1.
Pathways for the formation of PtdIns (3,4)P₂.

PTPase consensus	SCKSAKDRT/S
4-Phosphatase I	SCKSAKDRTAMS
4-Phosphatase II	CCKSAKDRTSMS
<i>C. elegans</i>	SCKSAKDRTAMS
Chicken	SCKSAKDRTAMS
Zebrafish	SCKSAKDRTAMS
Bovine	SCKSAKDRTAMS
Porcine	SCKSAKDRTAMS
Rat	SCKSAKDRTAMS

Figure 2.
Alignment of active sites of 4ptases.

Figure 3.

DNA sequences of mouse wild type 4-ptaseI (A) in the region surrounding the weeble mutation and a single nucleotide deletion in the weeble (B), indicated by an arrow. (C) Sequence from a mouse heterozygous for the weeble mutation. Note that following the single nucleotide deletion, sequence is obtained from both the wild type and mutant strands, yielding overlapping peaks on the chromatogram.

Figure 4.
Generation of radiation chimera mice.

Figure 5.
Blood flow traces from A-Wild type and B-weeble mice.