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Late signaling in the activated platelets upregulates tyrosine phosphatase SHP1 and impairs platelet adhesive functions: Regulation by calcium and *Src* kinase

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Abstract

Sustained stimulation of platelets with protease-activated receptor agonists in presence of extracellular calcium was associated with tyrosine dephosphorylation of specific proteins of relative mobilities 35, 67, and 75 kDa. From phosphatase assays and inhibitor studies SHP1, a *Src* homology 2 (SH2) domain-containing tyrosine phosphatase expressed abundantly in hemopoietic cells, was found to be upregulated in platelets between 25 and 30 min following thrombin stimulation. Concomitantly, SHP1 was tyrosine phosphorylated by, and coprecipitated with, *Src* tyrosine kinase. SHP1 activation, association with *Src* and dephosphorylation of specific proteins were dependent on extracellular calcium and maintenance of a higher cytosolic calcium plateau. There was progressive impairment of platelet functions like aggregability and clot retraction, associated with downregulation of fibrinogen-binding affinity of integrin $\alpha_{IIb}\beta_3$, in the platelets exposed to thrombin for 45 min. This could reflect the late physiological changes in platelets when the cells are consistently exposed to stimulatory signals under thrombogenic environment in vivo.

Keywords: Platelet activation; Intracellular calcium; Protein tyrosine phosphatase; *Src* tyrosine kinase; SHP1; Thrombin

1. Introduction

Under a thrombogenic environment platelets exhibit a range of hemostatic responses in vivo, including adherence to exposed endothelium, formation of cell–cell aggregates, release of stimulatory molecules from the intracellular stores and assembly of prothrombinase complex on surface membrane leading to generation of thrombin. Aggregated platelets remain embedded in a meshwork of fibrin plugging the site of injury. Platelets remaining free in the immediate vicinity are consistently exposed to the high titer of agonists in the microenvironment, get activated and subsequently incorporated in the growing thrombus. Presence of calcium in the extracellular milieu is a strong determinant in favor of thrombus formation and contributes to this ongoing cycle. Although platelet aggregation serves an important role in the control of hemorrhage, uncontrolled growth of thrombus leads to the pathological state [1]. Indeed, nature has provided several physiological 'checks' to contain the process, which include generation of NO and prostacyclins by the endothelium [2], inbuilt pathways for removal of thrombin, fibrin and platelets from the lesion site and other ill understood mechanisms.

Platelet activation is associated with temporal waves of protein tyrosine phosphorylation induced by the members of *Src* family kinases, Syk and focal adhesion kinase [3]. Under resting condition, the level of tyrosine phosphorylated proteins remains low in the platelets, suggestive of the existence of basal phosphatase activity [4]. [1]PTPs identified in platelets include PTP1B, *Src* homology 2 (SH2)-domain containing tyrosine phosphatases 1 and 2 (SHP1 and SHP2) and PTPH1 [4–6], out of which SHP1 is predominantly expressed in the hemopoietic cell lines. SHP1 contains tandem SH2 domains within the N-

Abbreviations: PTP, protein tyrosine phosphatase; PAR, protease-activated receptor; pNPP, para-nitrophenyl phosphate; FITC, fluorescein isothiocyanate; DMSO, dimethylsulphoxide; SH2, *Src* homology domain 2; BAPTA-AM, 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester); $[Ca^{2+}]_i$, intracellular-free calcium concentration

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terminus, enabling its association with tyrosine phosphorylated proteins including *Src* [7–9]. Enzymatic activity of SHP1 is upregulated by phosphorylation of its tyrosine residues [8,10], or its proteolytic cleavage by the calcium-dependent protease, calpain [11,12]. On the contrary, phosphorylation on Ser^{591} at its C-terminus by protein kinase Cα leads to inhibition of the phosphatase activity [13]. SHP1 is known to translocate to the cytoskeleton in activated platelets [14] and associate with the membrane protein PECAM1 (CD31) [15,16] and adaptor protein Grb2 [17]. Tyrosine phosphatases are implicated in different platelet functions like regulation of calcium influx [18], clot retraction [19], microvesicle shedding [11] and post-aggregatory signaling [20,21]. Indeed, a balance between tyrosine kinase and phosphatase activities is essential for platelet function.

Studies on platelet activation have mostly focused on the early signaling events in agonist-stimulated cells. However, in physiological milieu platelets remain continuously exposed to thrombin for a prolonged period at the site of thrombus. Thus, the aim in this paper was to investigate the effects of sustained thrombin exposure on the platelets.

2. Experimental procedures

2.1. Antibodies and chemicals

Monoclonal anti-phosphotyrosine IgG2b (clone PY99) and polyclonal antibody against SHP1 (sc-287) were purchased from Santa Cruz, USA. Fluorescein isothiocyanate (FITC)-labeled PAC1 and annexin V were from Becton Dickinson (USA). The horseradish peroxidase-labeled secondary antibodies were purchased from Bangalore Genei, India. BAPTA/AM (196419), PP1, PP3 and protein tyrosine phosphatase inhibitor-1 (α-Bromo-4-hydroxyacetophenone) (540200), a specific inhibitor for SHP-1 and PTP1B, were from Calbiochem, USA. Human thrombin, apyrase (grade III) (A 7646), EGTA, acetylsalicylic acid, bovine serum albumin (fraction V), HEPES, phenylarsine oxide (P-1646), sodium orthovanadate (S-6508), protein A–sepharose, Nonidet P-40, the protease inhibitors, dimethylsulphoxide, paraformaldehyde and *p*-nitrophenylphosphate were from Sigma, USA. Tween 20 was from Bio-Rad (USA). The Super Signal West Pico chemiluminescent substrate was from Pierce (USA). The reagents for electrophoresis were either from SRL (India) or Spectrochem (India). Fura-2 AM and anti-*Src* mAb (MAP 327) were kind gifts from Dr. Peter Presek (Martin-Luther-Universtät, Halle-Wittenberg). All other reagents were of analytical grade.

2.2. Platelet activation and aggregation

Platelets were isolated by differential centrifugation from fresh human blood donated by healthy volunteers, as already described [20,21]. The final cell count was adjusted to $0.8–1.2\times10^{9}$/ml. Platelets were stimulated with thrombin (1 U/ml) for 45 min at 37 °C in the absence of stirring in a Chrono-log platelet ionized calcium aggregometer (model 600). In some experiments, platelets were incubated with 2 mM Ca^{2+} for 90 s prior to addition of thrombin. Whenever indicated, EGTA (4 mM) was added simultaneously with thrombin. In other experiments, platelets were incubated at 37 °C with either PAO (2 μM), sodium orthovanadate (7.5 mM) or protein tyrosine phosphatase inhibitor-1 (50 μM) for 5 min each, or with BAPTA-AM (50 μM) for 20 min, prior to the addition of thrombin. Control platelets were incubated with respective vehicles at concentrations similar to the reagents; volume of DMSO did not exceed 0.5% of total volume in any of the experiments. Whenever required, aggregation was induced by stirring (1200 rpm) and measured from the change of light transmission. For analysis of protein phosphorylation, platelets were transferred to one-fourth volume of 5× sample buffer, supplemented with EGTA (5 mM), EDTA (5 mM), sodium orthovanadate (1 mM), and proteins were solubilized by boiling at 95 °C for 5 min.

2.3. Fibrin clot retraction

Clot retraction studies were carried out as described previously [22] with some modifications. Briefly, platelets were stimulated with thrombin for different time points and transferred into glass tubes containing equal volumes of autologous plasma. Fibrin clots were formed within a few minutes, which retracted progressively at 37 °C. After 1 h, the clots were carefully removed and the extent of retraction was determined from percent increase in the residual plasma volumes. Control experiments were carried out without platelets.

2.4. Measurement of intracellular calcium

PRP was incubated with 2 μM Fura 2-AM for 45 min at 37 °C in the dark. The Fura 2-loaded platelets were washed and resuspended in buffer B at 10^{8} cells/ml. Fluorescence was recorded in 400 μl aliquots of platelet suspension at 37 °C under non-stirring condition in a Hitachi fluorescence spectrophotometer (model F-2500). Excitation wavelengths were 340 and 380 nm and emission wavelength was set at 510 nm. Changes in intracellular free calcium concentration, $[Ca^{2+}]_i$, were monitored from the fluorescence ratio (340/380) using FL Solutions software. Fluorescence ratio was acquired continuously for 2 min and light path was blocked thereafter by closing the shutter in instrument to avoid photobleaching of the fluorochrome. $[Ca^{2+}]_i$ was measured subsequently in the same samples after 30- and 45-min intervals. Intracellular free calcium was calibrated according to the derivation of Grynkiewicz et al. [23].

2.5. Platelet tyrosine phosphatase assay

Assay for total platelet tyrosine phosphatase was carried out as described previously [24]. Briefly, platelets were lysed with 0.5 vol of 3× lysis buffer (3% NP-40, 150 mM Tris–HCl, pH 7.4, 450 mM NaCl, 3 mM PMSF, 15 μg/ml leupeptin and 15 μg/ml aprotinin) for 30 min at 4 °C. PTP activity was measured in 200 μl aliquots of platelet lysates incubated with equal vol of the reaction mixture (HEPES, 62 mM, pH 7.5 and *p*-nitrophenylphosphate, 8 mM) at 37 °C. Appearance of the cleaved product was monitored at 405 nm in a spectrophotometer (Pharmacia, model Ultrospec 1000E). Duplicates were performed in the presence of pervanadate (1 mM) to ensure that the measured phosphatase activity was due to tyrosine phosphatase. To determine the contribution of SHP-1, platelets were preincubated with protein tyrosine phosphatase inhibitor-1 (50 μM) for 5 min. In some experiments in vitro phosphatase assay was carried out on the immunoprecipitated SHP1 (see below).

2.6. Immunoprecipitation

Five hundred microliters platelet suspension (equivalent to about 1 mg platelet proteins) was lysed in equal volume of 2× radioimmunoprecipitation assay (RIPA) buffer (pH 7.4, 2% Triton X-100, 2 mM EGTA, 2 mM EDTA, 100 mM HEPES, 150 mM NaCl, 2 mM Na_3VO_4, 21 μM leupeptin, 2 mM phenylmethylsulfonyl fluoride, 20 μM pepstatin A and 0.56 trypsin inhibitor unit/ml aprotinin) on ice. The lysate was clarified by centrifugation at 16,000×*g* for 15 min at 4 °C. The supernatant was precleared with rabbit IgG and protein A–sepharose, followed by incubation with 2 μg anti-SHP1 antibody and protein A–sepharose together for 2 h. The immunoprecipitates were pelleted by centrifugation for 5 min at 16,000×*g* and washed thrice in 1× RIPA buffer (without protease inhibitors). Immune complexes were finally solubilized in 35 μl of 2× sample buffer.

2.7. Immunoblotting studies

Equal amounts of platelet proteins (80–100 μg) were resolved on 10% SDS-PAGE gels and electrophoretically transferred to Immobilon-P PVDF membranes (Millipore) by using NovaBlot semidry system (Amersham Biosciences) as per manufacture's instructions. The membranes were blocked with 10% bovine serum albumin in 10 mM Tris–HCl, 150 mM NaCl, pH 8.0 (TBS) containing 0.05% Tween 20 for 2 h at room temperature. The blots were then incubated for 2 h with different dilutions of primary antibodies as follows: anti phosphotyrosine mAb, 1:1000; anti-*Src* mAb, 1:500, and rabbit anti-SHP1, 1:500. Following three washing, the blots were incubated for 1 h with horseradish

peroxidase-labeled anti-mouse IgG or anti-rabbit IgG as required. The antibody binding was detected using enhanced chemiluminescence and quantified in an Agfa Duoscan T1200 flatbed scanner using GeneTools software (Syngene, UK). Whenever necessary, blots were stripped of primary and secondary antibodies by incubation for 10 min at 70 °C in the stripping buffer (TBS containing 0.05% Tween 20, 2% SDS and 1% 2-mercaptoethanol) [25,26] and reprobed.

2.8. Flow cytometry

Aliquots (45 μl) of washed platelets (1×10^8/ ml) were incubated at 37 °C for 1 min with 5 μl of FITC-PAC1 or FITC–annexin V, followed by treatment with thrombin (1 U/ml) for the specified periods. Isotype-matched control antibodies were used for FITC-PAC1 studies to rule out non-specific binding. Platelets were fixed with 100 μl of 1% paraformaldehyde dissolved in PBS (pH 7.4). Flow cytometry was carried out with a FACSCalibur (Becton Dickinson) flow cytometer acquiring data at 10,000 events per sample. The geometric mean fluorescence intensities in the stimulated and unstimulated samples were calculated.

2.9. Presentation of data

Data are presented as mean±S.E.M. of at least three individual experiments from different blood donors. Analysis of statistical significance was performed using Student's paired *t*-test and differences were considered significant when $p<0.05$. Immunoblots shown are representatives of at least four different experiments.

3. Results

3.1. Dephosphorylation of specific proteins on tyrosine in thrombin-stimulated platelets is regulated by extracellular calcium and is independent of integrin $\alpha_{IIb}\beta_3$

Platelets were incubated with thrombin (1 U/ml) under non-stirring condition for up to 45 min in the presence of 2 mM extracellular Ca^{2+}. Specific platelet proteins (of relative mobilities 35, 67 and 75 kDa) were phosphorylated on tyrosine following 2-min exposure to thrombin, which almost remained unchanged until 30 min. This was followed by significant dephosphorylation of these three proteins (by 39±3%, 32±2% and 39±3%, respectively, compared to the cells activated for 2 min; $p<0.05$, for all the three proteins) after 45 min of thrombin stimulation (Fig. 1A, left panel, and Fig 1B). To further exclude the contribution of outside-in signaling we preincubated the platelets with the tetrapeptide RGDS (0.5 mM), which prevents binding of fibrinogen to the integrin. Presence of RGDS did not alter the state of phosphorylation of these proteins (data not shown), thus ruling out possible role of integrin-mediated signaling inputs in the process of dephosphorylation.

In order to examine the role of Ca^{2+} in thrombin-induced dephosphorylation, BAPTA-AM (50 μM) and EGTA (4 mM) were used to chelate intracellular and extracellular Ca^{2+}, respectively. Presence of either EGTA alone, or EGTA combined with BAPTA-AM, partially prevented dephosphorylation of the specific proteins (Fig. 1A, right panel). The inhibition of dephosphorylation after 45 min of thrombin stimulation was reflected in an increase in the intensities of the 35, 67 and 75 kDa bands by 4±1%, 20±1% and 17±5% ($p<0.05$, for 67 and 75 kDa bands), respectively, in the EGTA-treated platelets and by 14±1%, 37±4% and 23±5% ($p<0.05$, for all three bands), respectively, in the cells treated together with EGTA and BAPTA, compared to the cells suspended in presence of calcium (Fig. 1C). This was indicative of a regulatory role of calcium in the process of dephosphorylation. Almost identical observations were made when platelets were stimulated with thrombin receptor-activating peptide (TRAP) (10 μM), another PAR agonist, but the extent of dephosphorylation was found to be less (not shown).

3.2. Protein tyrosine dephosphorylation in thrombin-stimulated platelets is associated with upregulation of SHP1

Total platelet PTP activity was estimated in the platelets stimulated with thrombin in the absence of stirring. Resting platelets were found to have significant level of basal PTP activity, which was attenuated by preincubation with pervanadate (1 mM), a non-specific phosphatase inhibitor (data not shown). Stimulation with thrombin for 2 min led to an increase in total phosphatase activity by 14±4% and 7±1%, respectively, in the platelets incubated with calcium and EGTA (Fig. 2A). When cells were exposed to thrombin either for 30 min or 45 min in presence of calcium, total PTP activity was increased by 17± 4% or 38±7% of basal activity, respectively. On the contrary, there was lesser increase in PTP activity beyond 2 min in the EGTA-treated platelets. PTP activity at 45 min was significantly lower in the presence of EGTA compared to that in the Ca^{2+}-treated platelets ($p<0.05$, Fig. 2A), implicating a role for extracellular Ca^{2+} in stimulation of PTP. To correlate PTP activity with dephosphorylation of specific platelet proteins, we studied the effect of phenylarsine oxide (2 μM) on protein tyrosine phosphorylation. The extents of phosphorylation of 35, 67, and 75 kDa peptides were found to be higher (by 44±2%, 42±2% and 44±3%, respectively; $p<0.05$, for all three bands) in the PAO-treated platelets stimulated for 45 min in presence of calcium (Fig. 2B), compared to those in the absence of PAO, implicating phosphatase activity with the observed dephosphorylation. Similar observations were made with sodium orthovanadate (7.5 mM), another pharmacologically independent inhibitor (data not shown).

SHP1 is a major PTP expressed in the platelets, which is activated in a Ca^{2+}-dependent manner in thrombin-stimulated cells [7,20]. To explore the role of SHP1, tyrosine phosphatase assay was carried out in the presence of protein tyrosine phosphatase inhibitor-1, a specific inhibitor of SHP1 [27] and PTP1B, in the calcium-treated platelets stimulated with thrombin. In preliminary studies optimum conditions for the activity of the inhibitor were determined. For the subsequent experiments platelets were subjected to 5 min preincubation with the inhibitor (50 μM), at which the enzymatic activity of the immunoprecipitated SHP1 was completely inhibited. The SHP1 inhibitor reduced total tyrosine phosphatase activity in the activated platelets at all the time points studied up to 45 min (Fig. 3A). However, the decrease was not significant in the resting platelets and in the cells stimulated for 2 min. The reduction in total phosphatase activity by SHP1 inhibitor was statistically significant when platelets were stimulated continuously for 30 min ($p<0.05$, $n=5$) or 45 min ($p<0.01$, $n=5$) with thrombin (Fig. 3A). SHP1 inhibitor elicited an increase in the

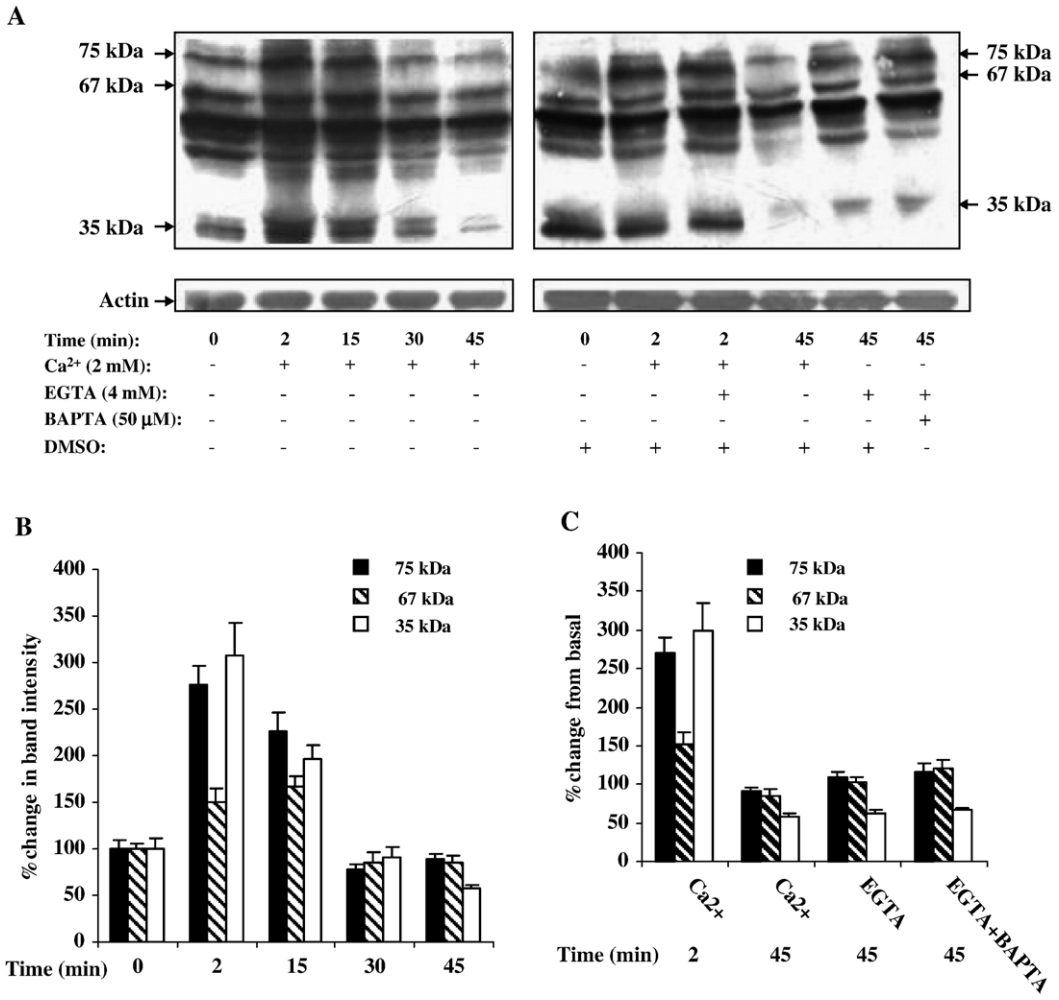

Fig. 1. Tyrosine dephosphorylation of platelet proteins following sustained stimulation with thrombin. Effect of extra- and intracellular calcium. (A) Platelets were activated with thrombin (1 U/ml) for different time intervals in the presence of Ca^{2+} (2 mM), EGTA (4 mM) or BAPTA-AM (50 μM) as indicated. Cells were lysed and immunoblotted against phosphotyrosine. The lowest panel shows the Coomassie staining of corresponding actin bands in the membrane to indicate equal loading in the lanes. The left and right panels represent two different experiments carried out on platelets from different individuals. Panels B and C represent densitometric analysis of the intensities of three specific bands (relative to corresponding actin bands) in the left and right panels of panel A, respectively. Data are presented as percent change in intensity of bands from the basal levels (mean±S.E.M, $n=3$).

phosphotyrosine contents of 35, 67 and 75 kDa peptides by 17±3, 40±4 and 41±3%, respectively ($p<0.05$, for all three bands), compared to those in its absence, in the platelets stimulated for 45 min (Fig. 3B). Thus, activation of SHP1 was temporally induced in the platelets exposed to thrombin for at least 30 min. As the inhibitor did not completely reverse the phosphatase activity to the basal level, PTPs other than SHP1 may also be implicated in the observed dephosphorylation. Subsequently, SHP1 was immunoprecipitated from platelets stimulated for different time points in presence of Ca^{2+} and in vitro phosphatase assay was carried out on the immune complex (Fig. 3C). The SHP1 activity was found to rise from its basal level in the unstimulated cells by about 8, 42 and 99%, respectively, following 2, 30 and 45 min of platelet stimulation.

To determine the tentative time point at which SHP1 gets activated in agonist-treated platelets, the phosphatase inhibitor was added at varying time intervals following thrombin stimulation, as indicated in the Fig. 4. Cells were lysed following 45 min of stimulation (Fig. 4, lanes 3–8). There was no change in the phosphotyrosine profile of the proteins when the SHP1 inhibitor was added either in the beginning or after 25 min of thrombin stimulation (lanes 4 and 5, respectively, Fig. 4). However, when the inhibitor was added after 30, 35 or 40 min following addition of thrombin, there was dephosphorylation of specific proteins (lanes 6, 7 and 8, respectively, Fig. 4), indicating prior activation of SHP1. Thus, activity of SHP1 was putatively upregulated between 25 and 30 min following exposure of platelets to thrombin.

3.3. Activated SHP1 gets tyrosine phosphorylated and associates with Src

Tyrosine phosphorylation of SHP1 by the *Src* kinase and regulation of *Src* activity by dephosphorylation induced by SHP1 are shown to be reciprocally regulated [28]. Since there was upregulation of SHP1 activity between 25 and 30 min of

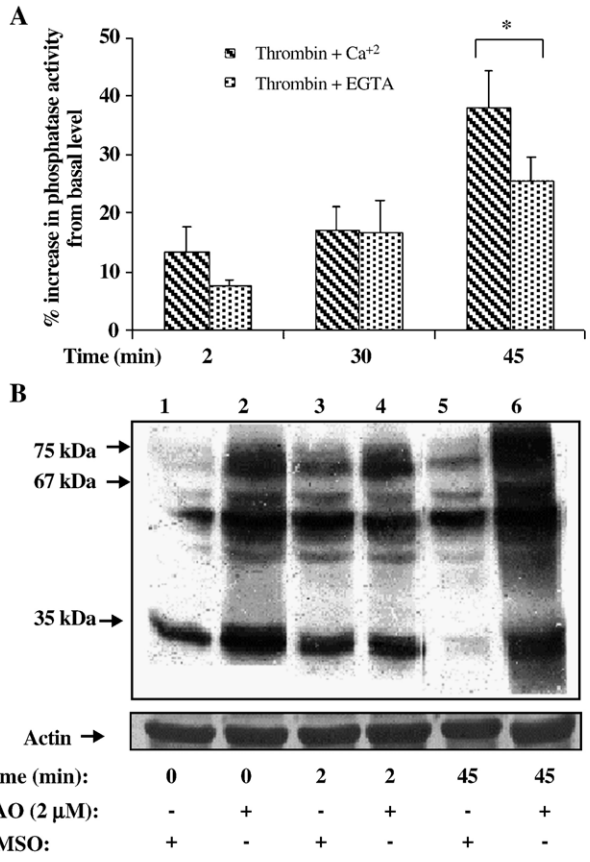

Fig. 2. Stimulation of protein tyrosine phosphatase activity in thrombin-treated platelets. (A) Platelets were stimulated with thrombin in presence of Ca^{2+} (2 mM) or EGTA(4 mM) without stirring. PTP activity in the cell lysates was measured from the generation of *p*-nitrophenolate from pNPP as described. Results are mean±S.E.M of five experiments (*$p<0.05$). (B) Washed platelets were activated with thrombin (1 U/ml) for varying time points in presence of Ca^{2+} as indicated. PAO (2 μM) or DMSO was added 5 min before agonist. Cells were lysed and immunoblotted against phosphotyrosine.

thrombin stimulation, we checked phosphotyrosine content of SHP1 and its association with *Src*. SHP1 was found to be phosphorylated on tyrosine (Fig. 5A) and co-immunoprecipitated with *Src* (Fig. 5B) following 30 and 45 min of thrombin stimulation in calcium-pretreated platelets. However, the amount of *Src* coprecipitated with SHP1 was about 3% of total, suggesting association of a fraction of cellular *Src* with SHP1. Immunoprecipitation of SHP1 in the platelets treated either with EGTA (4 mM) or PP1 (20 μM), a specific inhibitor of *Src*, prevented its association with *Src* (Fig. 5B) as well as tyrosine phosphorylation of SHP1 (data not shown), while PP3 (20 μM), the inactive analogue of PP1, had no effect (not shown), thus implicating a regulatory role for calcium and *Src* activity in SHP1 activation.

3.4. Level of intracellular calcium in thrombin-stimulated platelets is regulated by the presence of calcium in suspension buffer

Since dephosphorylation of specific platelet proteins was a function of extracellular calcium, we studied subsequently the changes in cytosolic calcium, $[Ca^{2+}]_i$, in the Fura 2-loaded platelets exposed to thrombin for 45 min. Cytosolic Ca^{2+} rose from the resting level of 52±6 nM to 375±13 nM within 10 s of thrombin stimulation in presence of 2 mM extracellular calcium ($n=5$) (Fig. 6A). The amplitude of transient $[Ca^{2+}]_i$ spike was less (297±10 nM) when extracellular calcium was chelated by EGTA, to be followed by a significant decrease in the Ca^{2+} plateau (Fig. 6A). The initial rise in calcium was mainly due to release from the intracellular stores, whereas maintenance of the cytosolic Ca^{2+} plateau could be attributed to influx from the extracellular source [18,29]. Levels of cytosolic Ca^{2+} following 30 and 45 min of thrombin exposure in the presence of calcium (134±15 and 158±

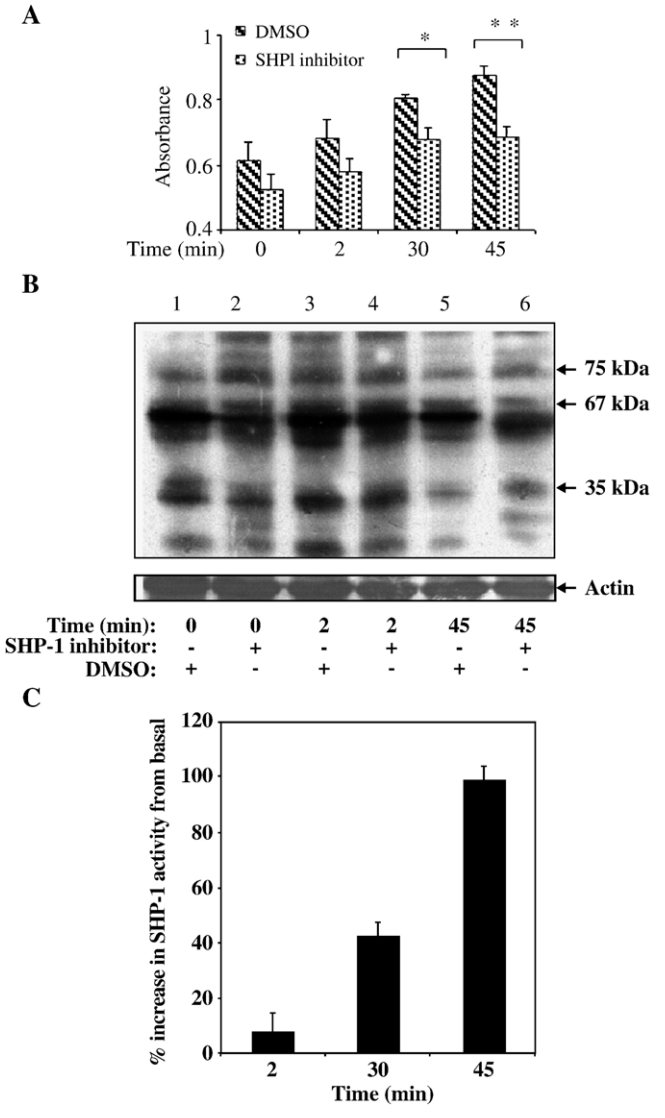

Fig. 3. Activity of SHP1 is upregulated in thrombin-stimulated platelets. Platelets were preincubated with either SHP1 inhibitor or DMSO and stimulated with thrombin for different time intervals. (A) Total platelet phosphatase activity was determined, as described in the legend to Fig. 2. Values are mean±S.E.M ($n=5$) (*$p<0.05$; **$p<0.01$). (B) Platelet proteins were immunoblotted against phosphotyrosine. (C) SHP1 was immunoprecipitated from platelets stimulated for different time points in presence of Ca^{2+}. In vitro phosphatase assay was carried out on the immune complex. The graph shows the percent increase in SHP1 activity from its basal level in unstimulated cells.

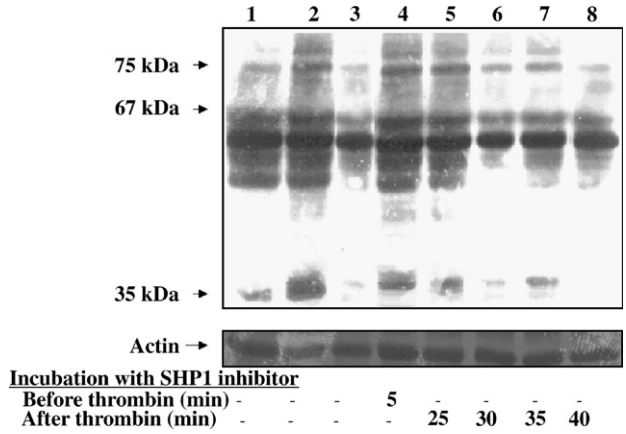

Fig. 4. Determination of temporal activation of SHP1 in thrombin-stimulated platelets. SHP1 inhibitor was added either 5 min before (lane 4) or 25, 30, 35 or 40 min after (lanes 5–8, respectively) the addition of thrombin. Platelets were lysed following 2 min (lane 2) or 45 min (lanes 3–8) after exposure to thrombin. Lane 1, resting platelets. Cell lysates were subjected to Western analysis and probed with anti-phosphotyrosine antibody. This is a representative of five experiments.

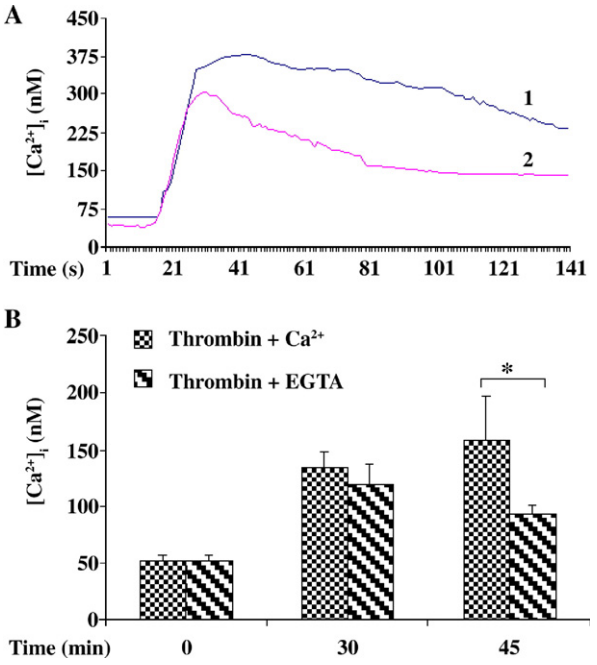

Fig. 6. Changes in cytosolic calcium in the platelets stimulated with thrombin for different time intervals. Effect of extracellular calcium and EGTA. (A) Fura-2-loaded platelets were stimulated with thrombin (1 U/ml) at 37 °C without stirring either in presence of Ca^{2+} (tracing 1) or EGTA (tracing 2). Changes in $[Ca^{2+}]_i$ were monitored with time as described under Results section. Fluorescence data were acquired as 340/380 ratio (emission 510 nm) as described under Experimental procedures. (B) Changes in $[Ca^{2+}]_i$ after 30 and 45 min of thrombin stimulation. Values are mean±S.E.M ($n=5$) (*$p<0.05$).

38 nM at 30 and 45 min, respectively) were found to be significantly higher compared to those under EGTA conditions (119±18 and 93±8 nM at 30 and 45 min respectively) (Fig. 6B).

3.5. Downregulation of fibrinogen-binding affinity of integrin $\alpha_{IIb}\beta_3$ following prolonged stimulation of platelets with thrombin

In order to understand the functional implications of signaling changes in platelets following sustained stimulation, extent of binding of PAC1, an antibody directed against the activation-specific epitope on integrin $\alpha_{IIb}\beta_3$, to platelets was studied under the experimental conditions. FITC-PAC1 binding was maximum at 2 min, which was significantly attenuated following 30 and 45 min of exposure to thrombin, thus signifying down-regulation of the fibrinogen-binding affinity in thrombin-treated platelets (Fig. 7A). To further substantiate this, platelet aggregation was induced by stirring the cells at 1200 rpm following 2, 30 and 45 min after thrombin addition. Aggregation was

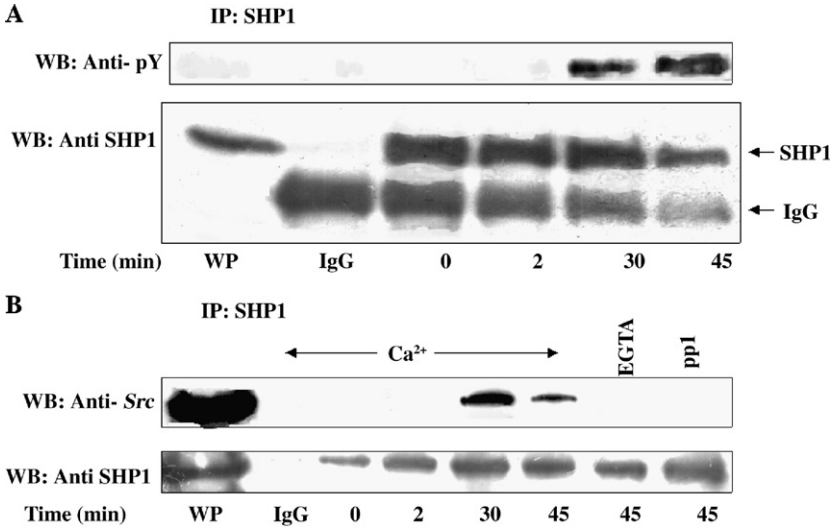

Fig. 5. SHP1 is tyrosine phosphorylated and coprecipitated with *Src* in a calcium-dependent manner. Platelets were stimulated with thrombin (1 U/ml) in presence of either calcium, EGTA or PP1 as indicated. SHP1 was immunoprecipitated and blotted against phosphotyrosine, *Src* or SHP1, as shown in the panels. The first and second lanes in each panel represent washed platelet lysates (WP) and controls (IgG-treated), respectively.

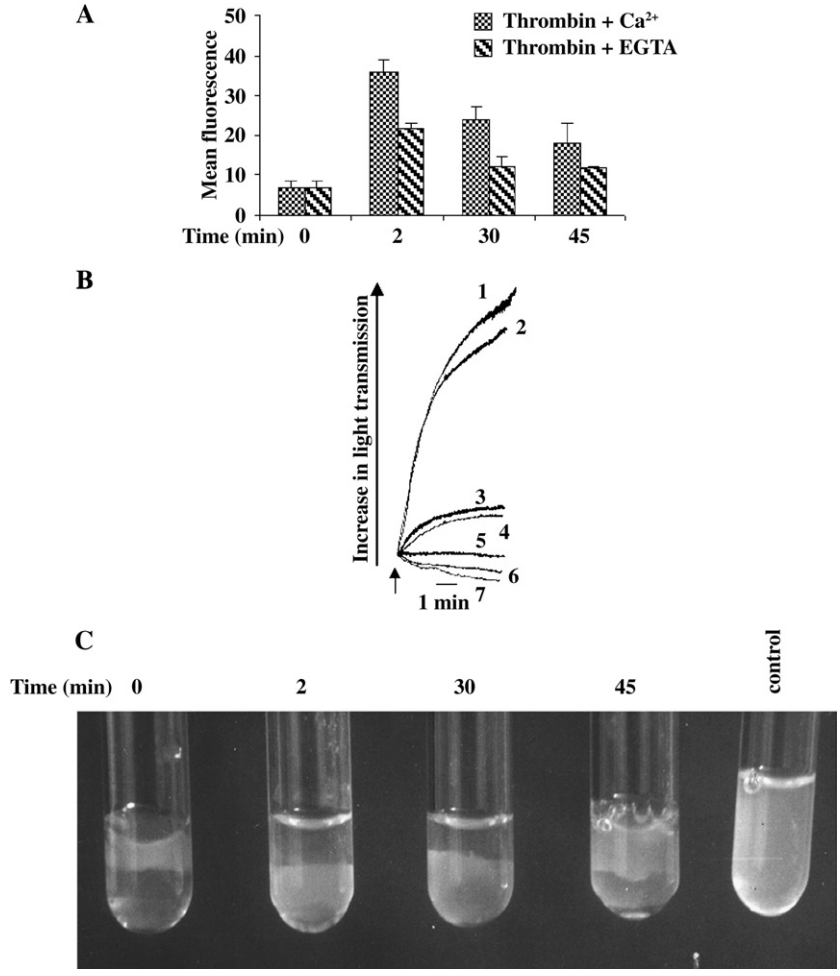

Fig. 7. Impairment of platelet functions following prolonged stimulation with thrombin. (A) Binding of FITC-PAC-1 to thrombin-stimulated platelets. Data are expressed as mean±S.E.M ($n=3$). (B) Platelets were activated with thrombin (1 U/ml) without stirring. At different time points aggregation was induced by stirring the cell suspension and increase in transmittance was recorded. The duration of activation without stirring was as follows: 2 min (tracings 2 and 4), 30 min (tracings 3 and 6) and 45 min (tracings 5 and 7). Studies were carried out either in presence of 2 mM Ca^{2+} (tracings 2, 3 and 5) or 4 mM EGTA (tracing 4, 6 and 7). Tracing 1 represents platelet aggregation in resting cells. Arrowhead indicates initiation of stirring. Data are representative of four different experiments. (C) Platelets were incubated with thrombin for different time periods as indicated. Clot formation was induced by addition of autologous plasma and allowed to retract for 1 h. The photograph shows the extents of retraction of fibrin clots under different conditions and is the representative of three different experiments.

significantly reduced after 30 min of thrombin addition in presence of Ca^{2+} and was completely obliterated after 45 min (Fig. 7B). In EGTA-treated platelets, the decrease in aggregation was more pronounced.

Ability to retract fibrin clot, another function mediated by the integrin $\alpha_{IIb}\beta_3$, was subsequently studied in the platelets exposed to thrombin in the presence of extracellular calcium. The extent of the retraction of the clots was recorded after 1 h of clot formation. Retraction was found to be progressively inhibited with the duration of exposure of the platelets to thrombin (Fig. 7C).

Calpain, the key protease activated during platelet aggregation [30] or storage [31], is responsible for proteolysis of several signaling and cytoskeletal proteins. Calpain activity was studied in the platelets stimulated with thrombin for 45 min in Coomassie-stained gels. No degradation of talin and filamin [31,32] was observed under above condition (data not shown), thus ruling out calpain activation. Externalization of PS to the outer platelet membrane leaflet, an activation response leading to generation of procoagulant surface [33], was subsequently studied from the extent of annexin V–FITC binding to the platelet membrane. The amount of PS on membrane outer leaflet was found to rise significantly within 2 min of thrombin stimulation in presence of calcium, which did not change significantly when platelets were continued to be stimulated for up to 45 min (data not shown). The data were suggestive of maintenance of stable procoagulant surface on platelet membrane during prolonged thrombin stimulation.

4. Discussion

In a physiological milieu of developing thrombus platelets remain continuously exposed to the agonists, including thrombin, released locally in the immediate microambience. Thrombin transmits its signals by ligation to its specific receptors, PAR-1 and PAR-4 [34] as well as to GpIb [35], leading to events like phosphoinositide turnover, rise in intracellular calcium, dramatic activation of several tyrosine and serine/

threonine kinases, and enhanced affinity towards adhesive ligands like fibrinogen. These intracellular changes take place very fast, starting from a few seconds after exposure of platelets to thrombin until up to 3–5 min. However, the late signaling events invoked in the thrombin-treated platelets, which would reflect the physiological changes in platelets in and around the growing thrombus in vivo, have not received significant research attention.

In the present study we have analyzed the temporal sequence of specific cellular events in platelets following sustained exposure to thrombin in presence of extracellular calcium. There was progressive impairment in the integrin-mediated events like clot retraction, stirring-induced platelet aggregation and fibrinogen-binding affinity of the integrin $\alpha_{IIb}\beta_3$. Calpain activity was not induced in these cells, in contrast to the earlier observations in aggregated platelets [30] or in the platelets aging in vitro [31]. As platelet activation is associated with upregulation of several protein tyrosine kinases, we studied the phosphotyrosine profile in thrombin-stimulated platelets in the presence of extracellular calcium. Three specific peptides (of relative mobilities 35, 67 and 75 kDa) were significantly dephosphorylated between 30 and 45 min of thrombin stimulation, indicative of late activation of putative tyrosine phosphatase(s). As aggregation was prevented by lack of stirring or inclusion of RGDS in the resuspension buffer, signaling inputs from the surface integrins were ruled out. Stimulation of platelets for 45 min exhibited a rise in total platelet PTP activity by about 40% compared to that in the resting cells. Dephosphorylation of proteins was not observed when cells were stimulated with collagen as the agonist (our unpublished observations), thus implicating PAR-mediated signaling in the dephosphorylation events.

To identify the candidate phosphatase(s) responsible for the observed dephosphorylation, we investigated the role of SHP1, the predominant PTP present in the platelets. Preincubation of the platelets with an inhibitor of SHP1 reduced total platelet phosphatase activity significantly following 30 and 45 min of thrombin exposure and significantly increased the phosphotyrosine contents of 35, 67 and 75 kDa peptides. SHP1 activity was enhanced from its basal level in the unstimulated cells by about 42 and 99%, respectively, following 30 and 45 min of platelet stimulation, indicating a temporal upregulation of platelet SHP1 activity at a late stage following exposure to thrombin. The 7% increase in SHP1 activity following stimulation of platelets for 2 min was not significant. From a time course experiment we determined the SHP1 activity to be induced between 25 and 30 min after the addition of thrombin. Although our data suggest a significant increase in SHP1 activity under the experimental condition, they do not rule out a possible contribution from PTP1B or another tyrosine phosphatase in the observed dephosphorylation.

To identify the factors regulating phosphatase activation we studied the association of SHP1 with the non-receptor tyrosine kinase, *Src*, in thrombin-treated platelets. The latter has been shown to activate SHP1 by phosphorylating it on tyrosine residues [28,36]. SHP1, in turn, regulates *Src* activity by dephosphorylating it on Y-530 [28]. Thus, activities of SHP1 and *Src* appear to be reciprocally regulated in the platelets. In our study SHP1 was found to be tyrosine phosphorylated after 30 min of thrombin stimulation, the extent of which was still higher after 45 min, consistent with the previous reports that phosphorylated SHP1 is enzymatically active [8,20,21]. *Src* co-immunoprecipitated with the phosphorylated SHP1 at 30- and 45-min time points in thrombin-activated platelets incubated in presence of calcium. PP1 (a specific inhibitor of *Src*) prevented tyrosine phosphorylation of SHP1 as well as its association with *Src*, thus implicating activated *Src* in the regulation of SHP1 activity.

From our data it was evident that, calcium present in the extracellular medium critically regulated tyrosine phosphatase activity in the thrombin-stimulated platelets. Capacitative entry of calcium from the extracellular milieu is responsible for the maintenance of intracellular Ca^{2+} plateau (after the initial spike) in the thrombin-treated platelets [18,29], although non-capacitative influx of Ca^{2+} has also been described in the platelets [37]. Tyrosine phosphatases have been implicated in the capacitative Ca^{2+} entry in platelets [18]. In the present study intracellular Ca^{2+} plateau remained significantly lowered at all the time points studied in the absence of calcium in suspension buffer. This was associated with lack of significant rise in platelet PTP activity. Tyrosine-specific dephosphorylation of the three proteins was, too, partially prevented. Interestingly, chelation of extracellular calcium precluded tyrosine phosphorylation SHP1 and its association with *Src* in the thrombin-treated platelets, which contrasted with the observation in presence of calcium, thus signifying a potential role of calcium in regulating SHP1 activation.

SHP1 has been proposed to play a constitutive role in maintaining low levels of protein tyrosine phosphorylation within hemopoietic cells including platelets, as evidenced from the early death of mice lacking active SHP1 (motheaten phenotype) [13]. SHP1 has also been shown to exert an inhibitory influence on cellular activation through its recruitment to phosphorylated immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and dephosphorylation of key signaling proteins in the activation cascade [7,38–40]. It is possible that SHP1 activity contributed towards functional impairment of platelets following sustained stimulation with thrombin, as reported in this study. SHP1 has also been functionally implicated in integrin-mediated platelet responses like clot retraction [19] and post-aggregation events [20,21]. However, SHP1 activation reported in the present study was integrin-independent as cells were not aggregated. Our data are consistent with the following model: platelets are incorporated into the growing hemostatic plug in vivo under a thrombogenic environment. Platelets remaining free in close proximity are continuously exposed to thrombin generated locally, leading to a sustained high intracellular Ca^{2+} plateau. The latter has a critical role in regulating activation of SHP1 at a temporally late stage (after 25–30 min under in vitro condition). *Src* activity appears to be stimulated consequent to the rise in $[Ca^{2+}]_i$ but upstream of SHP1 activation. The implication of dephosphorylation of the three specific peptides is not clear and the role of other putative tyrosine phosphatases cannot be ruled out from the above data. Functionally, platelets exposed to above situation exhibit decreased aggregability, associated with reduced fibrinogen-

binding affinity of integrin $\alpha_{IIb}\beta_3$. This would prevent incorporation of these platelets into the growing thrombus, which is an integrin-mediated event, and thus restrict the size of the thrombus. In parallel, steady PS exposure would ensure a procoagulant surface [33], or mark these cells for subsequent clearance by the macrophages [41].

During the process of thrombus formation the adjacent platelets ensure contact-dependent signaling through integrin $\alpha_{IIb}\beta_3$, Eph kinases/ephrins and CD40L/CD40, leading to stabilization of plug [42–44]. The growth of platelet plug is subsequently inhibited and restricted to the site of the vessel injury. Nitric oxide, ADPase and prostacyclins secreted from intact endothelium have all been implicated in confining the size of platelet plug [2]. The cGMP-PKG pathway in vWF and thrombin signaling through GPIb-IX, also induces a late inhibitory response that limits the size of platelet aggregates [45]. Thus, the signaling pathway described in the present report would contribute further to restrict incorporation of the free peripheral platelets into the thrombus, preventing further growth of thrombus and confining it to the site of the lesion.

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