

# G<sub>12/13</sub> Signaling Pathways Substitute for Integrin $\alpha$ IIb $\beta$ 3-Signaling for Thromboxane Generation in Platelets

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## Abstract

**Background:** We have previously shown that ADP-induced TXA<sub>2</sub> generation requires signaling from  $\alpha$ IIb $\beta$ 3 integrin in platelets. Here we observed that, unlike ADP, protease-activated receptor (PAR)-mediated TXA<sub>2</sub> generation occurs independently of  $\alpha$ IIb $\beta$ 3. PAR agonists, but not ADP, activate G<sub>12/13</sub> signaling pathways. Hence, we evaluated the role of these pathways in TXA<sub>2</sub> generation.

**Principal Findings:** Inhibition of ADP-induced thromboxane generation by fibrinogen receptor antagonist SC57101 was rescued by co-stimulation of G<sub>12/13</sub> pathways with YFLLRNP. This observation suggested an existence of a common signaling effector downstream of integrins and G<sub>12/13</sub> pathways. Hence, we evaluated role of three potential tyrosine kinases; c-Src, Syk and FAK (Focal Adhesion Kinase) that are known to be activated by integrins. c-Src and Syk kinase did not play a role in ADP-induced functional responses in platelets. Selective activation of G<sub>12/13</sub> pathways resulted in the activation of FAK, in the absence of integrin signaling. Interestingly,  $\alpha$ IIb $\beta$ 3-mediated FAK activation occurred in a Src family kinase (SFK)-independent manner whereas G<sub>12/13</sub> pathway caused FAK activation in a SFK and RhoA-dependent manner. A FAK selective inhibitor TAE-226, blocked TXA<sub>2</sub> generation. However, in comparison to WT mice, Pf4-Cre/Fak-Floxed mice did not show any difference in platelet TXA<sub>2</sub> generation.

**Conclusions:** Therefore, we conclude that differential activation of FAK occurs downstream of Integrins and G<sub>12/13</sub> pathways. However, the common effector molecule, possibly a tyrosine kinase downstream of integrins and G<sub>12/13</sub> pathways contributing to TXA<sub>2</sub> generation in platelets remains elusive.

**Citation:** Bhavaraju K, Lakhani PR, Dorsam RT, Jin J, Hitchcock IS, et al. (2011) G<sub>12/13</sub> Signaling Pathways Substitute for Integrin  $\alpha$ IIb $\beta$ 3-Signaling for Thromboxane Generation in Platelets. PLoS ONE 6(2): e16586. doi:10.1371/journal.pone.0016586

**Editor:** Christian Schulz, German Heart Center, Germany

**Received:** September 8, 2010; **Accepted:** December 21, 2010; **Published:** February 10, 2011

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**Funding:** This work is supported by HL81322, HL80444 and HL60683 from National Institutes of Health to SPK. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Platelet activation is an essential component of hemostasis and thrombosis, and involves engagement of complex signaling machinery. Injury to sub-endothelium results in platelet adhesion, and subsequent spreading on exposed collagen. Platelet activation also leads to reorganization of platelet cytoskeleton, release of granular contents from dense and alpha granules and finally culminates in integrin activation leading to platelet aggregation [1,2,3]. ADP released from the dense granules and the thromboxaneA<sub>2</sub> (TXA<sub>2</sub>) generated from activated platelets further act as positive feedback mediators and amplify the initial platelet responses and stabilize the hemostatic plug [4,5].

TXA<sub>2</sub> is generated from its precursor arachidonic acid through cyclooxygenase pathway [6]. TXA<sub>2</sub> acts on the TP (Prostanoid) receptors and recruits more platelets to the site of injury. Platelets can be activated by broad range of agonists, which can be further classified as strong or weak. ADP, serotonin, and epinephrine, are considered weak agonists [7] whereas thrombin, SFLLRNP (PAR1

agonist), AYPGKF (PAR4 agonist) [8], and Convulxin (GP VI agonist) [9] are strong agonists. Not only these agonists activate platelets with varied potencies but they also induce distinct signaling pathways. Previous studies have shown that ADP-induced TXA<sub>2</sub> generation in platelets is integrin-dependent [10] however; it is not known whether stronger agonists such as thrombin depend on integrin-mediated signaling for TXA<sub>2</sub> generation.

ADP activates two G-protein coupled receptors P2Y<sub>1</sub> and P2Y<sub>12</sub>, activating G<sub>q</sub> and G<sub>i</sub> pathways, respectively [1,3]. In platelets, neither of the ADP receptors can couple to G<sub>12/13</sub> proteins whereas PAR receptors couple to G<sub>12/13</sub> proteins [11]. G<sub>12/13</sub> pathways have been shown to activate Rho kinase [12] and Src family kinases (SFKs) [13]. Earlier studies have shown that G<sub>12/13</sub> pathways mediate calcium-independent shape change [12,14], and play a potentiating role in Akt phosphorylation [13] and dense granule secretion [15]. Co-stimulation of platelets with G<sub>12/13</sub> and G<sub>i</sub> also leads to platelet aggregation [16].

Agonist binding to platelet receptors results in complex intracellular signaling events termed as inside-out signaling, which

leads to the activation of integrins  $\alpha$ IIb $\beta$ 3 and  $\alpha$ 2 $\beta$ 1. Activated integrins change conformation and bind multivalent ligands such as fibrinogen and von Willebrand Factor (vWF) [17]. Signaling events from ligand binding to fibrinogen receptor are termed as outside-in signaling which, in turn regulate platelet adhesion, spreading, and clot retraction [18]. Outside-in signaling also causes phosphorylation of  $\beta$ <sub>3</sub> cytoplasmic tails [19,20], and activation of Phospholipase C (PLC  $\gamma$ ) [21], tyrosine kinases such as c-Src, Syk [21] and Focal adhesion kinase (FAK) [22]. FAK is a 125 kDa protein, expressed in both megakaryocytes and platelets [23]. FAK is tyrosine phosphorylated on six tyrosine residues viz., Y397, Y407, Y576/577, Y861 and Y925 [24].

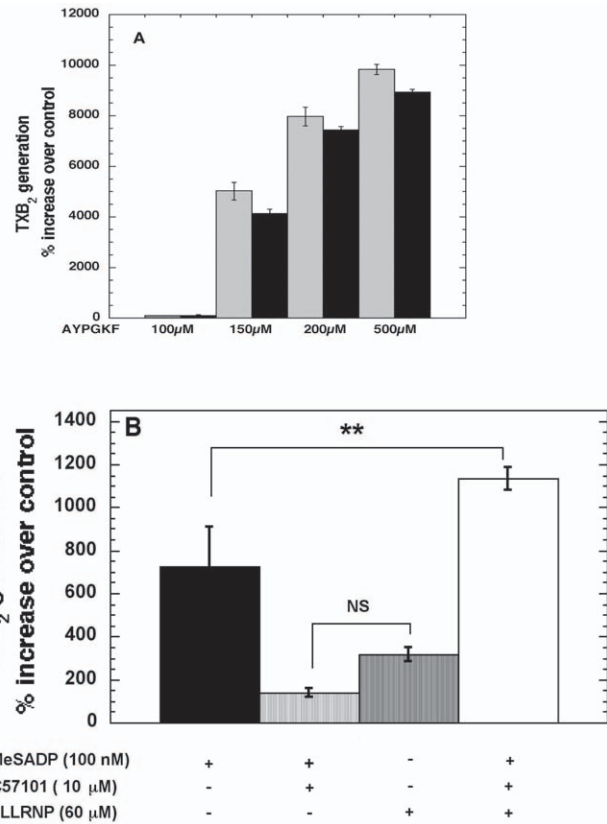
Other signaling molecules, involved in outside-in signaling are Protein Tyrosine Phosphatase-1B (PTP-1B) [25], Protein Phosphatase 1C (PP1c) [26], Calcium and integrin binding protein (CIB) [27] and Protein Kinase C- $\beta$  (PKC- $\beta$ ) [28,29].

In this study we show that, G<sub>12/13</sub> pathways, cause TXA<sub>2</sub> generation even when signaling from integrin is blocked. We present an evidence that c-Src and Syk kinase do not play a role in ADP-induced functional responses and FAK can be activated downstream of integrin  $\alpha$ IIb $\beta$ 3 as well as G<sub>12/13</sub> pathways. However, G<sub>12/13</sub>-mediated activation of FAK occurs via Rho kinase and SFKs, whereas integrin-mediated FAK activation is independent of SFKs. Studies to evaluate the role of FAK in thromboxane generation showed that FAK does not contribute to thromboxane generation downstream of integrins. In conclusion, neither c-Src, Syk nor FAK contribute to integrin-mediated thromboxane generation in platelets. Therefore, the common signaling molecule downstream of integrins and G<sub>12/13</sub> remains yet to be identified.

## Results

### Regulation of thromboxane generation in platelets by G<sub>12/13</sub> pathways

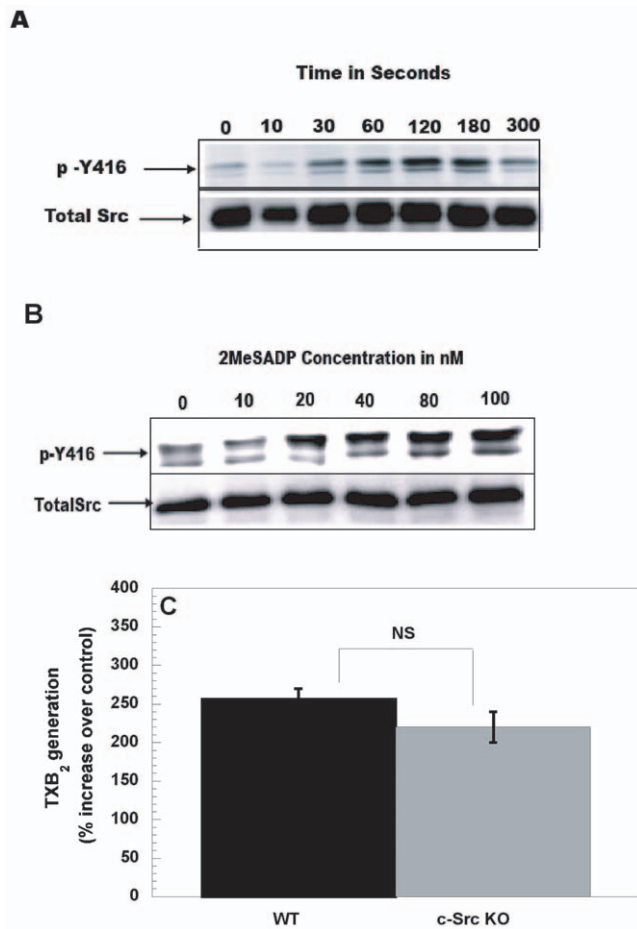
ADP-induced TXA<sub>2</sub> generation is dependent on integrin activation and as pre-treatment of platelets with fibrinogen receptor antagonist (SC57101) abrogated ADP-induced TXA<sub>2</sub> generation [10]. We investigated the effect of a fibrinogen receptor antagonist (SC57101) on PAR-mediated TXA<sub>2</sub> generation. Searle Research developed SC57101 (Skokie, IL) based on the RGDS structure. SC57101 and its analogs are fibrinogen receptor antagonists and do not affect inside out signaling [10]. As shown in Fig. 1A, varying concentrations of AYPGKF, a PAR4 agonist, caused TXA<sub>2</sub> generation in the presence or absence of SC57101, indicating that PAR agonists cause thromboxane generation independently of integrin signaling. One of the main signaling differences between ADP and PAR agonists is that only PAR agonists can activate G<sub>12/13</sub> pathways [11]. In order to evaluate the role of G<sub>12/13</sub> pathways in platelet TXA<sub>2</sub> generation we co-stimulated platelets with 2MeSADP and/or YFLLRNP. 2MeSADP is a more potent agonist than ADP at the P2Y1 and P2Y12 receptors [30] and YFLLRNP is a weak agonist of PAR1 that selectively activates G<sub>12/13</sub> pathways (at low doses) [16,31]. As shown in Fig. 1B, 2MeSADP-induced thromboxane generation was completely blocked in the presence of SC57101. YFLLRNP alone did not cause any significant TXA<sub>2</sub> generation in human platelets. However, co-stimulation of platelets with 2MeSADP and YFLLRNP in presence of SC57101 caused TXA<sub>2</sub> generation, indicating that the G<sub>12/13</sub> pathways can rescue the inhibition rendered by fibrinogen receptor antagonist. Hence, G<sub>12/13</sub> pathways can substitute for integrin-mediated signaling by probably activating similar effector molecules.



**Figure 1. Regulation of thromboxane generation by G<sub>12/13</sub> pathways.** Non-aspirin-treated, washed human platelets were stimulated with different concentrations of AYPGKF in presence and absence of SC57101 (10  $\mu$ M) (A) or other agonists (B) as indicated for 3.5 minutes at 37°C under stirring conditions (900 rpm) in an aggregometer. After 3.5 minutes the reaction was stopped by snap-freezing in dry ice-methanol bath. TXB<sub>2</sub> levels were analyzed as described in "Materials and Methods". The values are representative of 3 independent experiments mean  $\pm$  S.E.M (n=3). The data were analyzed by student t-test and ANOVA, \*\* P $\leq$ 0.005 was considered significant. doi:10.1371/journal.pone.0016586.g001

### Role of c-Src and Syk downstream of integrin $\alpha$ IIb $\beta$ 3 in thromboxane generation in platelets

Previous studies have shown that c-Src and Syk are signaling effectors in platelets that are regulated by integrins [32]. In order to identify the common signaling molecules downstream of fibrinogen receptor and G<sub>12/13</sub> pathways, we evaluated the role of these individual kinases in ADP-induced TXA<sub>2</sub> generation. We reasoned that as these tyrosine kinases are activated downstream of fibrinogen receptor and because ADP-induced thromboxane generation requires signaling events from fibrinogen receptor, at least one of these kinases could be crucial for ADP-induced thromboxane generation. 2MeSADP-induced phosphorylation of c-Src Y416 occurred in a time and concentration-dependent manner (Figs. 2A and B). However, 2MeSADP-induced TXA<sub>2</sub> generation in wild type mice was not different from the c-Src knockout mice (Fig. 2C), suggesting that either other Src family members may compensate for the absence of c-Src or that c-Src activation downstream of ADP receptors is not involved in TXA<sub>2</sub> generation. We then studied Syk kinase activation downstream of ADP receptors. Syk kinase was not activated downstream of ADP receptors (Fig. 2D), as determined by the phosphorylation of the Tyr525/526 residues [33]. Taken together, these results suggest,

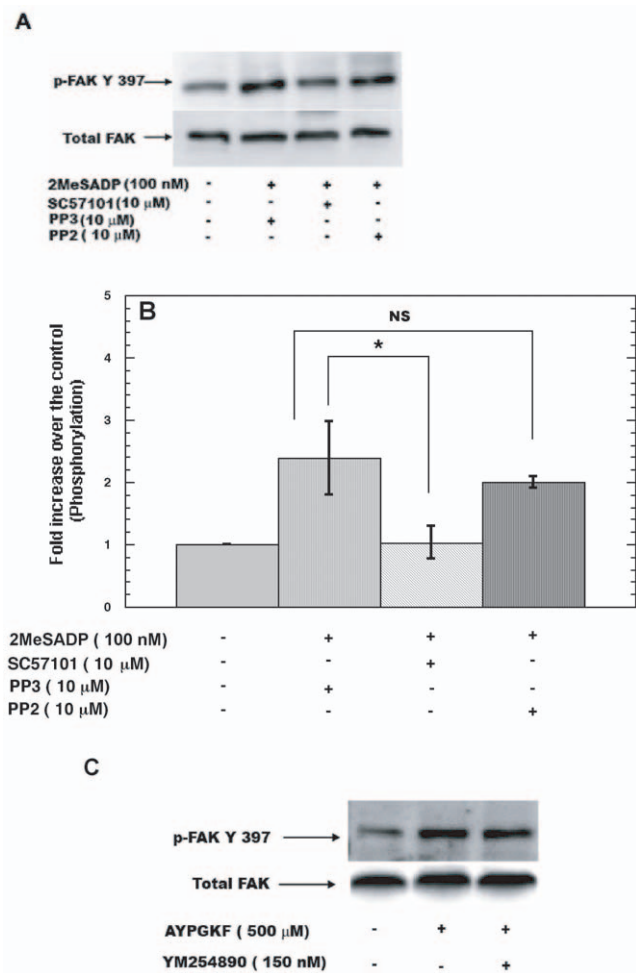


**Figure 2. Role of c-Src and Syk downstream of integrin  $\alpha$ IIb $\beta$ 3 in thromboxane generation in platelets.** Aspirin-treated, washed platelets were stimulated with 2MeSADP (100 nM) at various time points (A) or with varying concentrations of 2MeSADP (B) for one minute under stirring conditions at 37°C. The lysates were then subjected to western blotting analysis and probed with anti-phospho-(Y416) and total c-Src antibodies as lane loading control. Washed murine (WT or c-Src KO) platelets, without aspirin-treatment, were stimulated with 2MeSADP (100 nM) for 3.5 minutes and TXB<sub>2</sub> levels were analyzed (C) as described for Figure 1. The data are represented as the % Fold increase over the control. Aspirin-treated washed platelets were stimulated with 2MeSADP (100 nM) for (30–120 seconds) or convulxin (100 ng/ml) for 30 seconds under stirring conditions at 37°C (D). The lysates were then subjected to western blotting analysis and probed with anti-phospho-Syk(Y 525/526) and total Syk antibodies as lane loading control. The data are representative of at least 3 separate experiments.  
doi:10.1371/journal.pone.0016586.g002

that c-Src and Syk may not be essential for ADP-induced thromboxane generation in platelets.

### Focal Adhesion Kinase is activated downstream of integrins and G<sub>12/13</sub> pathways

We next evaluated the activation of FAK by ADP receptors, using Y397 phosphorylation as an activation marker. FAK contains multiple tyrosine phosphorylation sites and the sequential tyrosine phosphorylations of these sites causes complete FAK activation beginning with autophosphorylation Y397 phosphorylation [34]. As shown in Figs. 3A & B, FAK is activated downstream of ADP receptors and this activation is blocked by a fibrinogen receptor antagonist but not by a pan SFK inhibitor



**Figure 3. Focal Adhesion Kinase is activated downstream of integrins and G<sub>12/13</sub> pathways.** Aspirin treated, washed platelets were stimulated with 2MeSADP (100 nM) in presence or absence of reagents (as indicated) for 60 seconds under stirring conditions at 37°C (A). The lysates were then subjected to western blotting analysis and probed with anti-phospho-FAK (Y-397) and total FAK antibodies as lane loading control. The data are representative of mean  $\pm$  S.E.M (n = 3). The data was analyzed by ANOVA and \* P $\leq$ 0.05 was considered significant (B). Aspirin-treated washed platelets were stimulated with AYPGKF (500  $\mu$ M) in presence or absence of YM254890 (150 nM) (C), The lysates were then subjected to western blotting analysis and probed with anti-phospho-FAK (Y-397) and total FAK antibodies as lane loading control.  
doi:10.1371/journal.pone.0016586.g003

PP2. These results indicate FAK activation by ADP occurs in an integrin-clustering-dependent manner, independent of SFKs.

We further investigated FAK activation downstream of G<sub>12/13</sub> pathways. PAR agonists activate both G<sub>q</sub> and G<sub>12/13</sub> pathways in platelets [11]. The G<sub>q</sub> pathways can be inhibited by YM254890 [35] without affecting the G<sub>i</sub> or G<sub>12/13</sub> pathways [13]. Since YFLLRNP is a weak agonist for G<sub>12/13</sub> pathways at low concentrations and at higher concentrations can also activate G<sub>q</sub> pathways [16], we chose to stimulate platelets with AYPGKF (500  $\mu$ M) in the presence of YM254890 as tool for selective and strong activation of G<sub>12/13</sub> pathways for our phosphorylation studies [13]. As shown in Fig. 3C, AYPGKF caused activation of FAK in human platelets as determined by the phosphorylation of Y397 residue. This phosphorylation was unaffected in the presence of YM254890. It is important to note that 150 nM of

YM254890 abrogates AYPGKF-induced platelet aggregation and secretion [13] and hence autophosphorylation of FAK on Y397 is through G<sub>12/13</sub> pathways but not by outside-in signaling.

**Signaling pathways regulating FAK activation downstream of G<sub>12/13</sub> pathways**

We have shown that G<sub>12/13</sub> pathways activated by AYPGKF in the presence of YM254890 can activate FAK (Fig. 3A). Under these conditions we used pharmacological inhibitors to evaluate the signaling molecules that could regulate FAK phosphorylation. As shown in Figs. 4A&B, PP2, a pan SFK inhibitor abolished FAK phosphorylation mediated by G<sub>12/13</sub> pathways. Our results from Figs. 4C&D show that Rho kinase inhibitors H1152 and Y27632 also markedly inhibited G<sub>12/13</sub>-mediated FAK phosphorylation. These results indicate that both SFKs and Rho kinase play an important role in FAK activation downstream of G<sub>12/13</sub> pathways in platelets. In order to further delineate the signaling pathways downstream of G<sub>12/13</sub>, we also studied Src Y416 phosphorylation in presence of Rho kinase inhibitors. As shown in Figs. 4E&F, G<sub>12/13</sub>-mediated Src Y416 phosphorylation was dramatically inhibited in presence of Rho kinase inhibitors H1152 and Y27632, thus suggesting that SFKs are activated downstream of Rho kinase in the G<sub>12/13</sub> signaling cascade.

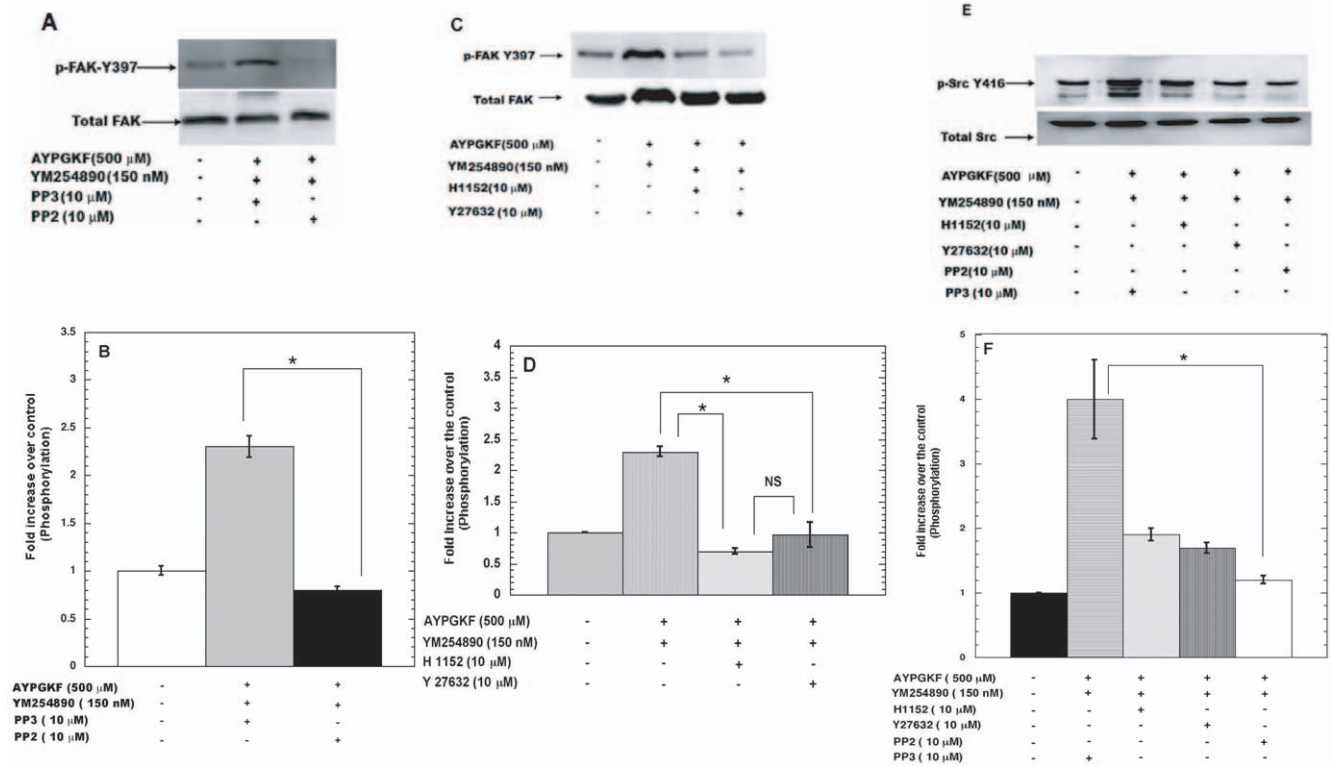
**Evaluation of FAK as a common signaling effector molecule regulating thromboxane generation downstream of integrins and G<sub>12/13</sub> pathways**

We next evaluated whether activated FAK played a role in ADP-induced TXA<sub>2</sub> generation. TAE-226 has recently been

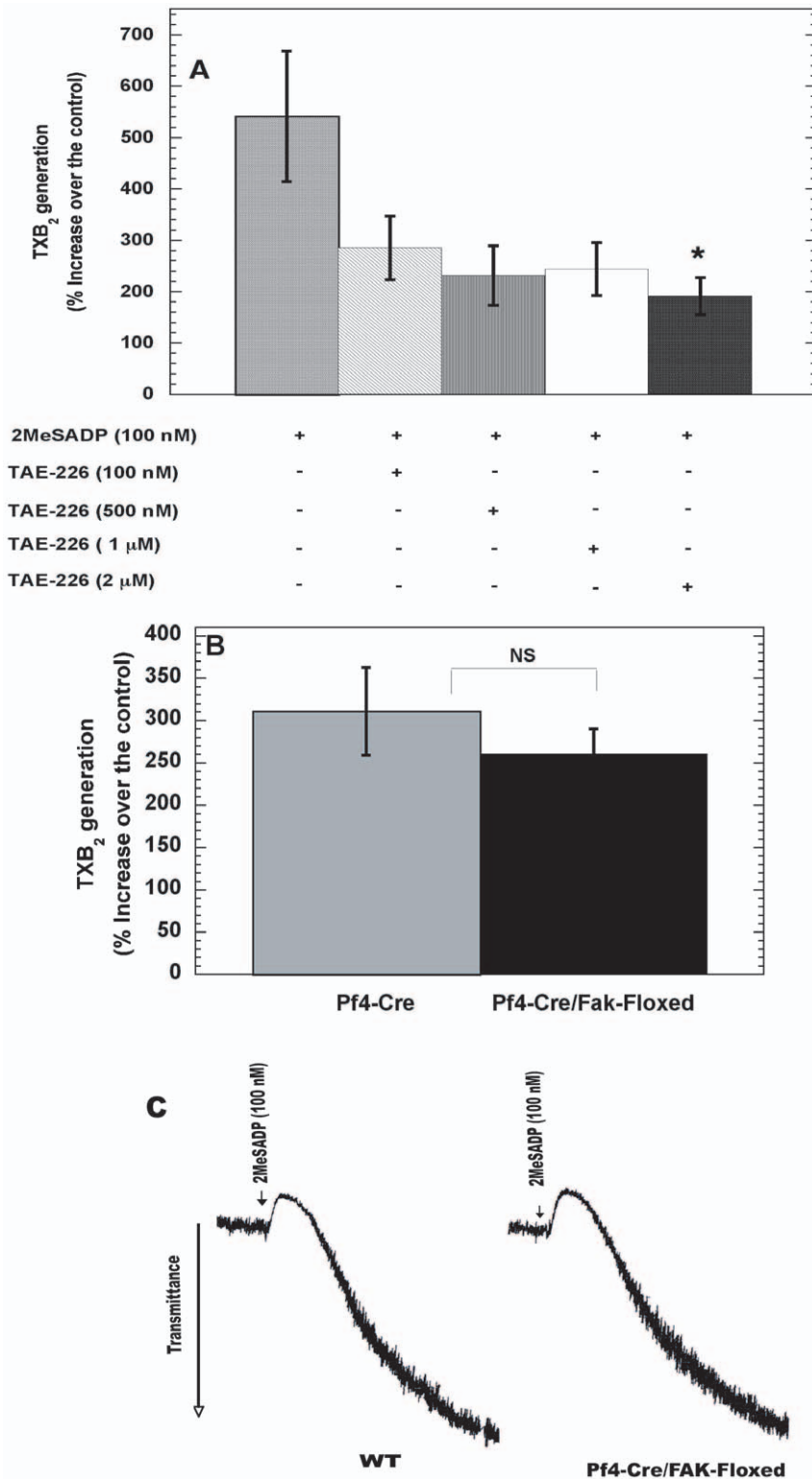
identified as a selective inhibitor of FAK with an IC<sub>50</sub> of 5.5 nM [36]. To determine the effect of FAK inhibition on ADP-induced thromboxane generation platelets were treated with varying concentrations of TAE-226. As demonstrated in Fig. 5A, TXA<sub>2</sub> generation was significantly inhibited by TAE-226 at a higher concentration of 2 μM. Pharmacological inhibitors are often known to have off target and broad-spectrum effects. The specificity of TAE-226 was never evaluated in platelets and reports suggest that TAE-226 inhibits Pyk2 (a Focal Adhesion kinase family member) with an IC<sub>50</sub> of 5 nM [37]. Hence, we studied thromboxane generation in WT and Pf4-Cre/FAK-floxed mice platelets. Murine platelets from WT and Pf4-Cre/FAK-floxed were stimulated with 100 nM of 2MeSADP and thromboxane levels were measured from WT and Pf4-Cre/FAK-floxed mice samples. As shown in Fig. 5B there was no significant difference observed thromboxane levels and aggregation tracings (Fig. 5C) between WT and Pf4-Cre/FAK-floxed. These results suggest that TAE-226 might exhibit some non-specific effects, and FAK is not the common signaling molecule regulating thromboxane generation downstream of integrins and G<sub>12/13</sub> pathways.

**Discussion**

Activated platelets release positive feedback mediators such as ADP and TXA<sub>2</sub>. The molecular mechanisms involved in thromboxane generation by different platelet agonists are not clearly understood. Weaker agonists such as ADP require outside-in signaling through activated fibrinogen receptor to cause thromboxane generation in platelets. Patients with Glanzmann’s



**Figure 4. Signaling pathways regulating FAK activation downstream of G<sub>12/13</sub> pathways.** Aspirin-treated, washed human platelets were pre-treated with different inhibitors (as indicated) for 5 minutes at 37°C followed by stimulation with AYPGKF (500 μM) for 90 seconds under stirring conditions at 37°C in an aggregometer. The lysates were then subjected to western blotting analysis and probed with anti-phospho FAK (Y-397) and total FAK antibodies as lane loading control (A & C) or anti-phospho-Src (416) and total c-Src antibodies as lane loading control (E). Quantitative data, normalized to the lane loading control, are representative of mean ± S.E.M (n=3). The data was analyzed by ANOVA and \* P≤0.05 was considered significant (B, D, and F). doi:10.1371/journal.pone.0016586.g004



**Figure 5. Evaluation of FAK as a common signaling effector molecule regulating thromboxane generation downstream of integrins and G<sub>12/13</sub> pathways.** Non-aspirin-treated, washed human platelets were pre-treated with varying concentrations of TAE-226 for 5 minutes at 37°C (A) and murine platelets from WT and Pf4-Cre/Fak-Floxed mice (B) were stimulated with 2MeSADP (100 nM) for 3.5 minutes and TXB<sub>2</sub> levels were analyzed as described for Figure 1. Aggregation tracings were measured from WT and Pf4-Cre/Fak-Floxed mice and representative tracings are shown (C). The values are representative of 3 independent experiments mean ± S.E.M (n=3). The data were analyzed by ANOVA and student t-test, \* P≤0.05 was considered significant. doi:10.1371/journal.pone.0016586.g005

thrombasthenia show defective thromboxane generation with ADP but not with thrombin [38]. Our results show that PAR agonists mediate TXA<sub>2</sub> generation independent of the integrin signaling. Previous studies by Stefanini *et al* [39] showed that stimulation of platelets with high and low doses of GPVI agonist convulxin in the presence of integrin blockers showed a marginal decrease in thromboxane generation, indicating that thromboxane generation downstream of GPVI receptors is not solely dependent on integrins. Thus, these results indicate that platelets can generate thromboxane in an integrin-dependent and-independent manner. Since PAR receptors can activate G<sub>12/13</sub> pathways we reasoned that there might be a common signaling molecule, which could be activated by both G<sub>12/13</sub> pathways (which are not activated by ADP) and integrins. This common signaling molecule might regulate TXA<sub>2</sub> generation downstream of G<sub>12/13</sub> pathways independent of integrins.

It has been known that several platelet agonists cause activation of G<sub>12/13</sub>, but not much is known about the intracellular signaling pathways downstream of this activation. Studies from our group have shown that G<sub>12/13</sub> pathways activate Src family kinases in platelets [13]. Klages *et al* [12] showed that G<sub>12/13</sub> proteins can activate tyrosine kinases such as Syk and c-Src. Interestingly these kinases are also known to be activated downstream of outside-in signaling [32]. Although integrins and G<sub>12/13</sub> pathways activate c-Src, it is clear that this kinase has no significant role in thromboxane generation downstream of ADP receptors (Fig. 2C). Interestingly, thromboxane A<sub>2</sub>, an agonist that couples to G<sub>q</sub> and G<sub>12/13</sub> [11], still caused the phosphorylation of p72Syk in G<sub>q</sub>-deficient mice [12]. This result suggests that activation of Syk might occur downstream of G<sub>12/13</sub> signaling. However, Syk does not appear to be activated downstream of ADP receptors (Fig. 2D). Hence, both c-Src and Syk can be ruled out as the common signaling molecule mediating thromboxane generation downstream of G<sub>12/13</sub> and integrin pathways.

Our studies show that FAK is differentially activated downstream integrins (Figs. 3A and B) and G<sub>12/13</sub> pathways (Figs. 3C, 4A and B). However, recently it was reported by Gong *et al* [40] that G<sub>13</sub> binds to integrin  $\alpha$ IIB $\beta$ 3 and mediates outside-in signaling. Gong *et al* claim that Rho activation downstream of PARs and integrins is temporally and spatially regulated and mediates opposing effects. With regards to FAK activation in platelets it does not seem to be the probable mechanism since ADP-induced FAK activation is independent of SFKs (Figs. 3A and B) and downstream of G<sub>12/13</sub> pathways FAK activation is SFK and Rho-dependent (Figs. 4A and 4C). Thus, there maybe some other integrin-mediated signaling pathways regulating FAK activation in platelets. Upstream of FAK, SFKs are activated by G<sub>12/13</sub> pathways [13]. SFKs regulate FAK activation downstream of G<sub>12/13</sub> pathways but not downstream of integrin signaling (Figs. 4A and 3A). Although integrin signaling leads to c-Src and FAK activation, our results suggest that Src/Syk pathways and FAK pathways are independently activated by integrins as outlined in Fig. 6, with possible differential functional implications in platelets.

G<sub>12/13</sub> pathways are known to activate RhoA/Rho kinase pathways leading to calcium-independent shape change [12,14]. These pathways are also known to regulate dense granule release through RhoA/Rho kinase pathways [15]. Our studies show that Rho kinase pathways regulate FAK activation through SFKs (Figs. 4E&F). This is a novel observation and indicates that Rho kinase pathways are the nodal point of SFK activation, ppIM $\delta$  phosphatase, and other pathways regulating dense granule release. We postulate that these pathways are independent of each other as; inhibition of SFKs (with PP2) has no significant effect on PAR-

mediated shape change or dense granule release in aspirin-treated platelets [41]. Hence, SFK activation occurs downstream, rather than upstream, of RhoA/Rho kinase pathways upon G<sub>12/13</sub> stimulation as outlined in Fig. 6. If SFK activation were to occur upstream of Rho kinase pathways, then SFK inhibition would have affected platelet shape change as well as dense granule release reaction in aspirin-treated platelets.

Furthermore, complementary approaches e.g. pharmacological FAK inhibitor TAE-226 and Pf4-Cre/Fak-Floxed mice were employed to evaluate role of FAK in thromboxane generation. Pre-treatment of platelets with various doses of TAE-226 did not lead to significant decrease in thromboxane levels. However, inhibition of thromboxane generation was observed at 2  $\mu$ M concentration only (Fig. 5A). Similarly thromboxane generation was not affected in Pf4-Cre/Fak-Floxed mice, when compared to WT littermates (Fig. 5B). These results indicate that FAK does not contribute to thromboxane generation downstream of integrins and TAE-226 might have some non-specific effects on platelets.

Since our data indicates that loss of FAK does not translate into diminished thromboxane generation, it is possible that some other tyrosine kinases might be compensating for the loss of FAK in platelets, however it might be unlikely since previous studies in Pf4-Cre/Fak-Floxed mice showed differences in tail bleeding times and platelet spreading and no upregulation of Pyk2 expression was observed in FAK-Floxed mice [23]. Recently, another FAK inhibitor PF-573,228 was shown to inhibit platelet aggregation [42]. However, our platelet aggregation studies comparing WT and Pf4-Cre/FAK-Floxed mice did not show any differences in aggregation (Fig. 5C). Furthermore, as our previous studies have shown that ERK can be activated even in the presence of integrin antagonists [43] therefore, ERK cannot be the common effector downstream of integrins and G<sub>12/13</sub> pathways.

Thus, we conclude that G<sub>12/13</sub> pathways through a Rho kinase/SFK dependent manner activate FAK. However, FAK activation downstream of integrins occurs independently of SFKs. Finally none of the three-tyrosine kinases c-Src, Syk or FAK seems to play a role in thromboxane generation downstream of integrins. Thus, the common signaling effector, possibly a tyrosine kinase, contributing to thromboxane downstream of G<sub>12/13</sub> pathways and integrins remains yet to be identified.

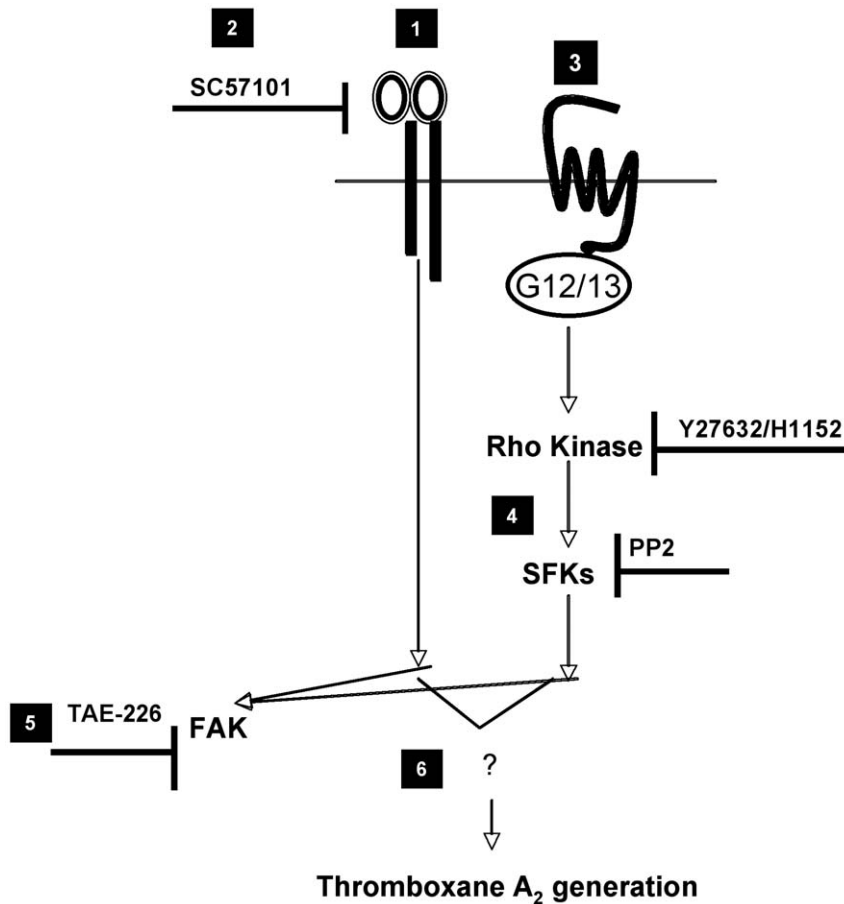
## Ethical Statement

Approval for this study was obtained from the Institutional Review Board of Temple University (Philadelphia, PA), and mice were used for physiological measurements using the protocol ID number 3364, approved by the Institutional Animal Care and Use Committee (IACUC).

## Materials and Methods

### Materials

2MeSADP, Apyrase grade VII, human fibrinogen, acetylsalicylic acid, were obtained from Sigma (St. Louis, MO). SC57101 was gift from Searle Research and Development (Skokie, IL). Hexapeptide AYPGKF was custom synthesized at Invitrogen (Carlsbad, CA). Convulxin was purchased from Centerchem Inc. (Norwalk, CT). The heptapeptide, YFLLRNP was synthesized by New England Biolabs (Beverly, MA) or by Research Genetics (Huntsville, AL). Phospho-specific antibodies against Y416 Src family, anti-Syk Y525/526, Total Src and Total Syk were obtained from Cell Signaling Technologies (Beverly, MA). Antibodies against FAK Y397 were obtained from Millipore, (Bedford, MA) and Total FAK was from Biosource (Camarillo, USA). PP2 and PP3 were purchased from Biomol (Plymouth



**Figure 6. Model depicting the regulation of TXA<sub>2</sub> generation by G<sub>12/13</sub> pathways and integrins through FAK.** Integrin clustering leads to FAK activation (1). Fibrinogen receptor antagonist SC57101 prevents integrin clustering hence inhibits FAK activation (2). FAK can be activated in an integrin-independent manner by G<sub>12/13</sub> pathways (3). FAK is activated downstream of Rho kinase and SFKs upon stimulation of G<sub>12/13</sub> pathways (4). FAK can be inhibited by TAE-226 (5). Common effector molecule downstream of integrins and G<sub>12/13</sub> pathways contributing to thromboxane generation are unknown (6).  
doi:10.1371/journal.pone.0016586.g006

Meeting, PA). The Rho kinase inhibitor H1152 was obtained from Toronto Research Chemicals (North York, ON). Y-27632 was obtained from Calbiochem (San Diego, CA). YM-254890 was a gift from Yamanouchi Pharmaceuticals Co., Ltd (Ibaraki, Japan). TAE-226 was generously provided by (Novartis Pharma AG, Switzerland). The other reagents were of reagent grade, and de-ionized water was used throughout.

### Animals

8–12 weeks old Pf4-Cre, and Pf4-Cre/FAK-floxed mice were generated in accordance with previously described protocol [44]. The generation of Src KO mice was described previously [45] and these mice along with wild type littermates in C57BL/6 background were used in the experiments.

### Preparation of washed human and murine platelets

All experiments with human volunteers were performed in accordance with Declaration of Helsinki. Whole blood was drawn from healthy, human volunteers selected from students, staff or workers at the Temple University with written informed consent. Donated blood was collected in tubes containing one-sixth volume of acid citrate dextrose (ACD) (2.5 g of sodium citrate, 1.5 g of citric acid, and 2 g of glucose in 100 ml of de-ionized water). Citrated blood was centrifuged and platelets were isolated with previously established protocol [46].

### Isolation of mouse platelets

Blood was collected from the vena cava of anaesthetized mice into syringes containing one-tenth blood volume of 3.8% sodium citrate as anticoagulant. Red blood cells were removed by centrifugation at 100 × g for 10 min. PRP was recovered, and platelets were pelleted at 400 × g for 10 min. The platelet pellet was resuspended in Tyrode's buffer (pH 7.4) containing 0.01 unit/ml apyrase. The isolated platelets were subsequently used for experiments.

### Western blot analysis

Aliquots of aspirin-treated, washed human platelets were lysed using Laemmli buffer in presence of dithiothreitol (DTT) (100 mM) and boiled for 10 min. The platelet lysates were loaded onto a 10% -Tris-glycine gel, subjected to SDS-PAGE (Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis), and transferred to PVDF membrane. Nonspecific binding sites were blocked by incubating the membrane in Tris-buffered saline-Tween (TBST; 20 mM Tris, 140 mM NaCl, 0.1% (vol/vol) Tween 20) containing 3% (wt/vol) bovine serum albumin (BSA) and 5% (vol/vol) Irish cream for 30 min at RT, followed by incubating it overnight at 4°C with gentle agitation in the primary antibody (1:1000 dilution for anti-FAK Y397, anti-Src Y416, anti-Syk Y525/526, anti-Syk, Anti-FAK and anti-Src in TBST with

3% BSA). After washing with TBST, the membranes were probed with an alkaline phosphatase-labeled secondary antibody (1:5000 dilutions in TBST with 3% BSA) for 1 hour at RT. After additional washing steps, membranes were incubated with chlordiazepoxide (CDP)-Star<sup>®</sup> chemiluminescent substrate (Tropix, Bedford, MA) for 10 min at RT, and immunoreactivity was detected using a Fuji Film Luminescent Image Analyzer (LAS-3000 CH; Tokyo, Japan).

### Measurement of TXA<sub>2</sub> generation in human and mice platelets

Washed human platelets (500  $\mu$ l brought to a concentration of  $2 \times 10^8$  platelets/mL) were stimulated with 2MeSADP (100 nM) in a lumi-aggregometer at 37°C with stirring at 900 rpm, in the presence or absence of TAE-226. Similarly murine platelets from WT and Pf4-Cre/FAK-floxed mice (250  $\mu$ l brought to a concentration of  $2 \times 10^8$  platelets/mL) were stimulated with 2MeSADP (100 nM). After 3.5 min of stimulation, the reaction was stopped by quickly freezing the sample in a dry ice-methanol

bath. The level of TXB<sub>2</sub>, the stable metabolite of TXA<sub>2</sub> was measured using a previously established protocol [47].

### Statistical Analysis

The results were quantified, expressed as mean  $\pm$  S.E.M. The data was statistically analyzed using Student's t-test and ANOVA. \*P $\leq$ 0.05/ \*\* P $\leq$ 0.05 were considered significant.

### Acknowledgments

We would like to thank Dr. Abdel Karim Sabri, Department of Anatomy and Cell Biology, and Dr. James L. Daniel, Department of Pharmacology, Temple University Medical School, for their helpful suggestions.

### Author Contributions

Conceived and designed the experiments: KB ISH AS SPK. Performed the experiments: KB PRL RTD JJ. Analyzed the data: KB JJ SPK. Contributed reagents/materials/analysis tools: ISH AS. Wrote the paper: KB SPK.

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