ADP-Stimulated Activation of Akt During Integrin Outside-In Signaling Promotes Platelet Spreading by Inhibiting Glycogen Synthase Kinase-3β

Kelly A. O’Brien, T. Kent Gartner, Nissim Hay, Xiaoping Du

Objective—Integrins mediate platelet adhesion and transmit outside-in signals leading to platelet spreading. Phosphoinositide 3-kinases (PI3Ks) play a critical role in outside-in signaling and platelet spreading; however, the mechanisms of PI3K activation and function in outside-in signaling are unclear. We sought to determine the role of the Akt family of serine/threonine kinases and activation mechanisms of the PI3K/Akt pathway in outside-in signaling.

Methods and Results—Akt inhibitors and Akt3 knockout inhibited platelet spreading on fibrinogen, indicating that Akt is important in integrin outside-in signaling. Akt inhibitors and Akt3 knockout also diminished integrin-dependent phosphorylation of glycogen synthase kinase-3β. Inhibition of glycogen synthase kinase-3β reversed the inhibitory effects of Akt3 knockout and inhibitors of Akt or PI3K on platelet spreading, indicating that glycogen synthase kinase-3β is a downstream target of Akt in outside-in signaling. Integrin-dependent activation of the PI3K-Akt pathway requires Src family kinase. Akt phosphorylation is also significantly inhibited in ADP receptor P2Y12 knockout platelets and further inhibited in P2Y12 knockout platelets treated with a P2Y1 antagonist. Consistently, P2Y12 knockout and P2Y1 inhibition together reduced platelet spreading.

Conclusion—These results demonstrate that integrin outside-in signaling and platelet spreading requires Src family kinase–dependent and ADP receptor–amplified activation of the PI3K-Akt-GSK-3β pathway. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: platelet • integrin • Akt • outside-in signaling • ADP
integrin ligation. However, the functional importance of Akt in mediating integrin outside-in signaling remains unknown.

In the present study, we address the following questions with regard to PI3K-Akt signaling during integrin outside-in signaling: (1) What is the upstream mechanism that activates the PI3K-Akt signaling pathway? (2) Whether and how Akt isoforms play a role as downstream mediators of PI3K signaling? and (3) If Akt is important in integrin signaling, what is the downstream mechanism mediating the PI3K-Akt signaling pathway? Here, we show that Akt isoforms are important in mediating PI3K signaling induced by integrin outside-in signaling. We further show that a downstream mechanism important in mediating the PI3K-Akt signaling is the Akt-mediated phosphorylation and inhibition of glycogen synthase kinase-3β (GSK-3β). In addition, we have discovered that the integrin-mediated full activation of the PI3K/Akt/GSK-3β signaling pathway requires the ADP receptor P2Y12 and involves P2Y1. These findings provide evidence for an important role of an SFK-dependent and ADP-amplified PI3K/Akt/GSK-3β signaling pathway in integrin outside-in signaling leading to platelet spreading.

Materials and Methods

Materials

Src family kinase inhibitor PP2, Akt inhibitor AktX, and PI3K inhibitors LY294002 and wortmannin were purchased from Calbiochem. P2Y12 antagonist 2-MeSAMP (2-methylthioadenosine 5′-monophosphate triethylammonium salt), P2Y1 agonist A23187 (adenosine-3′-phosphate-5-phosphate), BSA, and GSK-3β inhibitor SB216763 were purchased from Sigma. Fibrinogen was purchased from Enzyme Research Laboratories.

Animals

The generation of Akt1, Akt2, or Akt3 knockout mice were previously described. Mice used in this study were 8- to 15-weeks-old on C57BL/6 background. Animal usage and protocol was approved by the Institutional Animal Care Committee of the University of Illinois at Chicago.

Preparation of Platelets

For studies using human platelets, fresh blood was drawn by venipuncture from healthy volunteers and anticoagulated with one-seventh volume of acid-citrate-dextrose, as previously described. Institutional Review Board approval was obtained from the University of Illinois at Chicago, and informed consent was provided according to the Declaration of Helsinki. For the preparation of mouse platelets, fresh blood was drawn from mouse inferior vena cava and anticoagulated with acid-citrate-dextrose, as previously described. Blood from 5 to 6 mice of either genotype were pooled and platelets were isolated by differential centrifugation of whole blood with 0.1 μg/mL prostaglandin E1 and 1 U/mL apyrase (Sigma-Aldrich). Platelets were washed twice with modified Tyrode buffer containing 2% SD5, 0.1 mol/L Tris, 2% glycerol, 2 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L sodium orthovanadate, 2 mmol/L NaF, and complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Proteins were separated by SDS-PAGE on a 4% to 15% polyacrylamide gel and then transferred to polyvinylidene difluoride membranes. The membranes were immunoblotted with an antibody recognizing GSK-3β phosphorylated at Ser9, an antibody recognizing Akt (all isoforms) phosphorylated at Ser473, or antibodies against GSK-3β (Cell Signaling Technology, Danvers, MA), or Akt (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) (nonphosphorylated and phosphorylated).

Platelet Spreading on Immobilized Fibrinogen

Microscope cover glasses (Fisher Scientific, Waltham MA) were coated with 100 μg/mL fibrinogen (Enzyme Research Laboratories) in 0.1 mol/L NaHCO3 (pH 8.3) and blocked with 5% BSA in PBS. Washed mouse platelets (2×107 per mL) were allowed to adhere and spread on fibrinogen-coated wells (300 μL per well) at 37°C for 2 hours. Washed human platelets (1×107 per mL) were allowed to adhere and spread on fibrinogen-coated wells (300 μL/well) at 37°C for 1 hour. Slides were aspirated to remove nonadherent platelets and fixed with 4% paraformaldehyde, permeabilized, and stained with Alexa Fluor fluorescein isothiocyanate-conjugated phalloidin (Invitrogen, Carlsbad, CA), as previously described. Adherent platelets were observed with a Leica DM IRB fluorescence microscope (Leica Microsystems, Wetzlar, Germany) using ×100/1.30 NA oil objective (×1.5 magnification factor for mouse platelets, ×1.0 for human platelets). Images were acquired using a Cool SNAP HQ CCD camera (Photometrics) and processed with RS Image version 1.4 software (Photometrics) or Micro-Manager version 1.4.

A TP Secretion

Black, 96-well plates were coated with 100 μg/mL fibrinogen or BSA in 0.1 mol/L NaHCO3 (pH 8.3). Plates were blocked with BSA and washed human platelets (100 μL of 3×106 per mL) were added to fibrinogen-coated wells or BSA-(control) coated wells at 37°C for various time points. ENLITEN luciferase reagent (Promega) was added to wells and luminescence was immediately measured using Wallac Victor2 1420 multilabel counter (Perkin Elmger).

Results

The Role of Akt Isoforms as Downstream Effectors of PI3K in Mediating Platelet Spreading

We sought to determine whether the PI3K effector, Akt, plays an important role in integrin outside-in signaling. To inhibit the functions of all Akt isoforms, a pan Akt inhibitor, AktX, was used. AktX is a phenoxazine that selectively inhibits the phosphorylation of Akt, thus suppressing its kinase reactivity. This Akt inhibitor is not a phosphoinositide analog and thus does not directly affect functions of other phosphoinositide-regulated proteins. This excludes the possible nonspecific inhibition of other PI3K effectors, which is a potential complication with previously used Akt inhibitors, such as SH-6, a competitive inhibitor of phosphoinositide binding to Akt. Human platelets were treated with increasing doses of AktX and allowed to spread on immobilized fibrinogen. AktX dose-dependently inhibited human platelet spreading (Figure 1A and 1B). At 10 μmol/L, AktX completely abolished platelet spreading on fibrinogen. A similar dose-dependent defect in spreading was observed using mouse platelets treated with increasing concentrations of AktX (Figure 1C and 1D), suggesting that platelets from humans and mice similarly require Akt to promote integrin outside-in signaling and platelet spreading. To confirm the effectiveness of AktX on Akt activation and function in our experimental system,
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Wild-type (WT) mouse platelets were treated with increasing doses of AktX and allowed to spread on fibrinogen. After 30 minutes of spreading, platelets were harvested for Western blot analysis of Akt phosphorylation at Ser473. Akt is phosphorylated after platelet spreading on a fibrinogen-coated surface, indicating that Akt becomes activated during integrin outside-in signaling. AktX dose dependently inhibited phosphorylation of Akt at Ser473 (Figure 1E). Altogether, these data suggest Akt is activated and plays an important role in mediating human and mouse platelet spreading.

Platelets express 3 different Akt isoforms: Akt1, Akt2, and Akt3. Our recent results suggest that Akt3 is a major Akt isoform in platelets (≈70% of total Akt in mouse platelets).21 We, therefore, evaluated whether Akt3 plays a role in the integrin outside-in signaling pathway. Figure 2A shows that Akt is robustly phosphorylated in WT platelets spread on a fibrinogen-coated surface as detected by an anti-phospho-Akt S473 antibody recognizing all 3 Akt isoforms. Integrin-dependent phosphorylation of Akt was significantly inhibited in Akt3−/− platelets, indicating that Akt3 is a major Akt isoform phosphorylated downstream of integrin signaling in mouse platelets (Figure 2A). Phosphorylation of Akt at T308 was also significantly reduced in Akt3−/− mouse platelets adherent on fibrinogen (Figure 2A). Thus, we further investigated the role of Akt3 in platelet spreading using Akt3−/− mice. As shown in Figure 2B and 2C, Akt3−/− partially but significantly reduced platelet spreading on fibrinogen, suggesting that Akt3 is important in promoting platelet spreading. However, the incomplete inhibition by Akt3−/− is in contrast to the complete inhibition of platelet spreading by a higher dose of AktX, which inhibits all Akt isoforms. We also assessed the spreading of Akt1−/− or Akt2−/− mouse platelets. Akt1−/− mouse platelets showed a partial reduction in spreading compared with WT, whereas no difference was observed using Akt2−/− mouse platelets (Figure 1A and 1B in the online-only Data Supplement). Combined with the data obtained with pan-Akt inhibitors, our results suggest that Akt3 is an important isoform mediating integrin outside-in signaling, but other isoforms of Akt, particularly Akt1, may also be involved in mediating integrin outside-in signaling.

Akt-Dependent Phosphorylation of GSK-3β is a Downstream Mechanism of Integrin Outside-In Signaling

Akt is known to phosphorylate GSK-3β, which negatively regulates GSK-3β function.30 GSK-3β was shown to negatively regulate thrombin receptor–mediated platelet activation and in vivo thrombosis.31 To determine whether Akt3 mediates integrin outside-in signaling and platelet spreading by phosphorylating and inactivating GSK-3β, WT, and Akt3−/− platelets were allowed to spread on fibrinogen, and phosphorylation of Ser9 of GSK-3β was assessed by immunoblot with an antibody specifically recognizing phosphorylation at Ser9 site. Compared with WT platelets spread on fibrinogen, Akt3−/− platelets showed a significant reduction in GSK-3β phosphorylation, indicating that Akt3 is an important isoform mediating integrin outside-in signaling (Figure 2A). Akt1−/− mouse platelets also showed a trend of reduced GSK-3β phosphorylation but was not statistically significant (Figure 1C and 1D in the online-only Data Supplement). In order to further investigate whether negative regulation of GSK-3β is important in promoting integrin-dependent platelet spreading, we assessed...
the effect of GSK-3β selective inhibitor SB216763 on platelet spreading on fibrinogen-coated surfaces. Treatment of platelets with SB216763 significantly promoted platelet spreading compared with dimethyl sulfoxide control, indicating that GSK-3β negatively regulates platelet spreading and thus, inhibition of GSK-3β promotes platelet spreading (Figure 2B and 2C). Because Akt3−/− has reduced GSK-3β phosphorylation, which presumably results in increased GSK-3β activity, we sought to determine whether the spreading defect of Akt3−/− platelets could be corrected by treatment with GSK-3β inhibitor SB216763. Akt3−/− platelets were treated with the GSK-3β inhibitor and allowed to spread on fibrinogen. Indeed, the inhibitory effect of Akt3 knockout on platelet spreading was reversed by GSK-3β inhibitor SB216763 (Figure 2B and 2C). Interestingly, spreading of platelets treated with the pan-Akt inhibitor AktX or with the PI3K inhibitor LY294002 was partially rescued by treatment with SB216763 (Figure 3A and 3B). Thus, negative regulation of GSK-3β is a likely mechanism downstream of PI3K and Akt3 that promotes integrin–dependent platelet spreading, although we do not exclude possible additional mechanisms, particularly for other Akt isoforms.

Src and ADP Receptor–Dependent Activation of the PI3K-Akt Signaling Pathway During Integrin Signaling

Thus far, our experiments reveal an important role for the PI3K-Akt-GSK-3β signaling pathway in promoting platelet spreading. To understand the molecular mechanisms that are responsible for the activation of this pathway during integrin outside-in signaling, we examined whether phosphorylation of Akt can be affected by inhibitors of intracellular signaling molecules involved in integrin outside-in signaling. Adherent or control platelets were solubilized and analyzed by Western blot. A, Wild-type (WT) or Akt3−/− mouse platelets were plated on fibrinogen for indicated lengths of time or kept in suspension. Adherent or control platelets were solubilized and analyzed by Western blot. B, Platelets from Akt3−/− or WT mice were preincubated with GSK-3β inhibitor SB216763 or control dimethyl sulfoxide, plated on fibrinogen for 2 hours, fixed, permeabilized, stained, and observed with a fluorescence microscope. C, The surface area of single platelets in B was measured (mean±SE). Difference between groups was analyzed using Student t test. Shown in figure are representative pictures from 1 of the 3 experiments. Number of platelets analyzed is indicated above the bars.

Figure 2. Effects of Akt3 knockout on integrin outside-in signaling and the role of glycogen synthase kinase-3β (GSK-3β). A, Wild-type (WT) or Akt3−/− mouse platelets were plated on fibrinogen for 15, 30, or 60 minutes. B, Platelets from Akt3−/− or WT mice were preincubated with GSK-3β inhibitor SB216763 or control dimethyl sulfoxide, plated on fibrinogen for 2 hours, fixed, permeabilized, stained, and observed with a fluorescence microscope. C, The surface area of single platelets was measured (mean±SE). Difference between groups was analyzed using Student t test. Shown in figure are representative pictures from 1 of the 3 experiments. Number of platelets analyzed is indicated above the bars.

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Interestingly, integrin–mediated Akt phosphorylation was dramatically reduced in human platelets by an antagonist of ADP receptor P2Y12, 2-MeSAMP, and was further reduced when 2-MeSAMP was used together with A3P5P, an
antagonist of the other platelet ADP receptor, P2Y1 (Figure 5A). To exclude the possible nonspecific effects of ADP receptor antagonists, we also examined integrin-dependent Akt phosphorylation in mouse platelets deficient in the P2Y12 receptor (P2Y12 knockout). Indeed, Akt phosphorylation in P2Y12−/− platelets was also dramatically reduced (Figure 5B). P2Y12−/− mouse platelets treated with A3P5P further inhibited phosphorylation of Akt, as well as GSK-3β (Figure 5C). Thus, we conclude that ADP receptor signaling, particularly P2Y12 signaling, is important in integrin-induced and Src-dependent activation of the PI3K/Akt signaling pathway. It is important to note that there is still residual phosphorylation of Akt and GSK-3β in the presence of P2Y1/P2Y12 blockade, indicating a small pool of Akt and GSK-3β may be activated by ADP-independent mechanisms.

To directly address whether integrin ligation with fibrinogen induces dense granule secretion, we measured ATP release of platelets spread on fibrinogen using a luciferase assay. There was a significant increase in ATP release that was detected in platelets spread on fibrinogen, compared with platelets added to BSA control wells (Figure 5D). Integrin-dependent platelet adhesion does not require prior stimulation by exogenous agonists; therefore, our data indicate that ADP receptor signaling that stimulates activation of the PI3K/Akt pathway is likely to be induced by endogenously secreted ADP during platelet spreading on integrin ligands.

To determine whether the effects of ADP receptor signaling on PI3K/Akt pathway is functionally important in integrin-dependent platelet spreading, we compared the spreading of P2Y12−/− platelets and P2Y12 antagonist 2-MeSAMP–treated platelets with WT platelets. P2Y12−/− platelets and P2Y12 antagonist–treated platelets showed a significant but partial inhibition of platelet spreading compared with control platelets, indicating that P2Y12 signaling is important in promoting integrin outside-in signaling leading to platelet spreading (Figure 6A–6D). We also tested the effect of the P2Y1 antagonist A3P5P on spreading of control or P2Y12−/− mouse platelets. Consistent with the relatively minor defects caused by A3P5P in the activation of the PI3K-Akt pathway, only a minor spreading defect was observed in wild-type or human platelets treated with A3P5P. However, A3P5P significantly further inhibited platelet spreading in P2Y12−/− mouse platelets (Figure 6A and 6B). Human platelets treated with ADP receptor P2Y12 antagonist 2-MeSAMP (and to a much less degree A3P5P) similarly inhibited platelet spreading on fibrinogen (Figure 6C and 6D). These results suggest an important role for P2Y12 and P2Y1 receptors in promoting integrin outside-in signaling leading to platelet spreading.

Discussion

In this study, we show that (1) Akt, as a downstream effector of PI3K, plays an important role in mediating integrin outside-in signaling leading to platelet spreading; (2) Akt promotes platelet spreading by phosphorylating and inhibiting GSK-3β; and (3) Activation of PI3K-Akt-GSK-3β pathway is downstream from SFK and requires platelet secretion of ADP and ADP receptor signaling. Taken together, our results reveal a novel outside-in signaling pathway involving sequential activation of Src, ADP secretion, P2Y12/P2Y1-dependent activation of PI3K and Akt, and Akt-mediated inhibition of GSK-3β, leading to stimulation of platelet spreading (Figure 6E). Our study also demonstrates a novel mutually amplifying relationship between the G-protein–coupled P2Y12/P2Y1 receptor signaling pathways and integrin outside-in signaling pathways.
It has been shown by several groups that PI3Ks, including both type I and type II, play an important role in integrin signaling. However, the downstream effector that is important in mediating PI3K signals promoting platelet spreading is unclear. Among potential PI3K effectors, all 3 Akt isoforms have been shown to be expressed in platelets and to be important in mediating platelet granule secretion and secretion-dependent amplification of platelet aggregation. However, it is unclear whether Akt isoforms are also important in integrin outside-in signaling. In this study, we have shown that treatment of human or mouse platelets with a pan-Akt inhibitor, AktX, that is a nonphosphoinositide competitor, potently inhibits platelet spreading on fibrinogen (Figure 1). Furthermore, the spreading of Akt3−/− platelets was also partially inhibited (Figure 2B and 2C). Therefore, we conclude that Akt is an important downstream effector that mediates PI3K–dependent integrin outside-in signaling.

Our data further indicate that the Akt3 isoform is important in outside-in signaling but also suggest the involvement of other Akt isoforms.

Our data suggest that phosphorylation and inhibition of GSK-3β, a known Akt substrate, is an important downstream mechanism responsible for the role of Akt3 in promoting platelet spreading. This is supported by the data that...

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**Figure 4.** Upstream molecules important in Akt activation. Human platelets were treated with phosphoinositide 3-kinase inhibitor wortmannin (100 nmol/L) (A), PI3K inhibitor LY294002 (20 μmol/L) (B), Src family kinase inhibitor PP2 (10 μmol/L) (C), and Syk inhibitor picatannol (10 μmol/L) (D), or dimethyl sulfoxide (DMSO) control, and plated on immobilized fibrinogen or kept in suspension, solubilized and Western blot analysis was performed. p-GSK-3β indicates phospho–glycogen synthase kinase-3β.

**Figure 5.** Effect of P2Y12 knockout and ADP receptor antagonists on integrin–dependent Akt activation. A, Human platelets were preincubated with 2-methylthioadenosine 5′-monophosphate triethylammonium salt (2-MeSAMP) (50 μmol/L), adenosine-3′-phosphate-5′phosphate (A3P5P) (0.5 mmol/L), both, or control and plated on immobilized fibrinogen for 30 minutes or kept in suspension, solubilized, and analyzed by western blot. B, Wild-type (WT) or P2Y12−/− mouse platelets spread on fibrinogen and were analyzed by Western blot as in A. C, WT or P2Y12−/− mouse platelets were treated with A3P5P (0.5 mmol/L) or control and spread on fibrinogen for 30 minutes or kept in suspension. Western blot was performed as in A. D, Human platelets were added to BSA or fibrinogen-coated wells for indicated times. Luciferase was added and ATP secretion was detected using a luminometer. The relative quantity of ATP secretion is expressed as the percentage increase of BSA control (mean±SE, n=3). The difference between control and fibrinogen at 15- and 30-minute time points is significant (P<0.03), as determined by Student t test. GSK3β indicates phospho–glycogen synthase kinase-3β.
Akt3−/− platelets spread on fibrinogen showed a significant reduction in GSK-3β phosphorylation (Figure 2A) and that GSK-3β inhibition by SB216763 was sufficient to completely rescue the defect in Akt3−/− platelet spreading. These data indicate that the role of Akt3 in platelet spreading is mainly mediated by GSK-3β (Figure 2B and 2C). Consistently, inhibition of GSK-3β also partially rescued spreading defects of platelets treated with pan-Akt inhibitor AktX or PI3K inhibitor LY294002 (Figure 3A and 3B). However, partial rescue by SB216763 suggests the possible presence of other integrin–dependent Akt–mediated signaling pathways that are independent of GSK-3β. Further study is needed to resolve how GSK-3β negatively regulates platelet spreading.

It is known that ADP secretion can promote platelet spreading on immobilized fibrinogen and scavengers of ADP, such as apyrase, can inhibit platelet spreading.33,34 It has also been reported that platelets from a patient deficient in ADP receptor P2Y12 have defective spreading on fibrinogen.35 Accordingly, our data demonstrating defective spreading in P2Y12−/− mouse platelets and P2Y12 or P2Y1 inhibitor–treated platelets support the implication of ADP in amplifying integrin outside-in signaling and spreading (Figure 6). However, the mechanism by which ADP promotes platelet spreading has been unclear. It is possible that the roles of ADP can be a result of both increasing either integrin activation (inside-out signaling) or outside-in signaling. Although we do not exclude the well-established role for ADP in inside-out signaling and integrin activation, our data clearly show that 1 mechanism by which ADP promotes platelet spreading is its amplification of the PI3K-Akt-GSK-3β signaling pathway in integrin–mediated platelet spreading. SFK indicates Src family kinases; GSK-3β, glycogen synthase kinase-3β.

Figure 6. Effect of P2Y12 knockout and ADP receptor antagonists on platelet spreading. A, Wild-type (WT) or P2Y12−/− mouse platelets were treated with adenosine-3′,5′-diphosphate (A3P5P) (0.5 mmol/L) or control and spread on fibrinogen for 2 hours, fixed, permeabilized, stained, and observed, as previously described. Representative pictures. B, The surface areas of individual platelets were measured (mean±SE). Difference between groups was analyzed using Student t test. Number of platelets analyzed is indicated above the bars. Data are from 3 experiments. C, Human platelets were treated with 2-methylthioadenosine 5′-monophosphate triethylammonium salt (2-MeSAMP) (50 μmol/L), A3P5P (0.5 mmol/L), both, or control and spread on fibrinogen for 1 hour as described in A. D, Quantitation of C performed as in B. E, A schematic depicting the regulatory mechanisms and roles of the phosphoinositide 3-kinase (PI3K)/Akt pathway in integrin–mediated platelet spreading. SFK indicates Src family kinases; GSK-3β, glycogen synthase kinase-3β.
pathway during outside-in signaling (Figure 5). It is important to note that because integrin–dependent platelet adhesion does not require prior stimulation by agonists and because we have used carefully prepared resting platelets without exogenous agonists, ADP receptor signaling that stimulates activation of the PI3K/Akt pathway is likely to be induced by endogenously secreted ADP during platelet spreading on integrin ligands. Indeed, we, for the first time, detected granule secretion from platelets adherent on a fibrinogen-coated surface (Figure 5D). Thus, we conclude that granule secretion induced by integrin outside-in signaling plays an important role in stimulating activation of the PI3K/Akt pathway and thus platelet spreading.

Interestingly, our data also show that although ADP receptors play a major role in PI3K/Akt activation, activation of a small pool of Akt is independent of ADP receptors but clearly requires the function of SFKs (Figure 4C). The spreading defect in platelets treated with a high dose of AktX (Figure 1) also appears to be greater than that in P2Y12−/− platelets treated with P2Y1 antagonists (Figure 6), which is consistent with an ADP-independent activation of Akt in promoting spreading. In other platelet signaling pathways, such as thrombin receptor and GPIb-IX pathways, it has previously been shown that SFKs are important in stimulating the activation of PI3K/Akt pathway.36–38 Furthermore, in the platelet activation pathways induced by thrombin and thromboxane A2, PI3K/Akt activation involves a P2Y12-independent mechanism that accounts for a small fraction of Akt activation and a major amplification role of P2Y12/G1 signaling.47 Thus, a small, initial SFK-dependent activation of PI3K/Akt and an ADP-dependent amplification of PI3K/Akt activation may be a general mechanism in which the PI3K/Akt pathway becomes fully functional during platelet activation induced by not only soluble agonist receptors but also integrins and other adhesion receptors. It is interesting to note that a major role of Akt isoforms in regulating platelet function downstream from soluble agonists is to promote platelet dense granule secretion.18–21 Thus, we hypothesized that one of the roles of SFK–dependent initial activation of Akt isoforms in regulating platelet spreading may be to induce platelet dense granule secretion. Secreted ADP then greatly augments the PI3K-Akt-GSK-3β pathway, resulting in stronger amplification of integrin outside-in signaling and enhancement of platelet spreading. Nonetheless, the precise mechanism by which the PI3K-Akt-GSK-3β pathway promotes outward movement of the platelet membrane and spreading requires further examination.

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Disclosures
None.

References


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Supplemental Figure I. Spreading of Akt isoform knockout mouse platelets. (A) WT, Akt1−/−, Akt2−/−, or Akt3−/− mouse platelets (2x10^7/mL) were plated on immobilized fibrinogen for 2 hours, fixed, permeabilized, stained with phalloidin, and observed with a fluorescence microscope. Representative pictures are shown. (B) The surface area of single platelets was quantitated (average surface area ± SE). Statistical significance of the difference between groups was analyzed using Student’s t test. Numbers of platelets analyzed for each group (from three experiments) are indicated above the bars (*p<0.05). (C) WT or Akt1−/− mouse platelets were plated on fibrinogen for indicated time or kept in suspension as a control. Adherent platelets or control platelets were analyzed by Western blot for phosphorylation of GSK-3β at S9, and total GSK-3β levels. (D) Western blots from 3 experiments were scanned and quantified using NIH Image J for uncalibrated optical density. The relative quantity of phospho-GSK-3β of Akt1−/− vs. WT platelets spread on fibrinogen is expressed as the % of WT (mean ± SE). No statistical significance was obtained.