An important role for Akt3 in platelet activation and thrombosis

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The Akt family of serine/threonine kinases includes Akt1, Akt2, and Akt3 isoforms. Prior studies have reported that Akt1 and Akt2, but not Akt3, are expressed in platelets. Here, we show that Akt3 is expressed in substantial amounts in platelets. Akt3−/− mouse platelets selectively exhibit impaired platelet aggregation and secretion in response to low concentrations of thrombin receptor agonists and thromboxane A₂ (TXA₂), but not collagen or VWF. In contrast, platelets from Akt1−/− or Akt2−/− mice are defective in platelet activation induced by thrombin, TXA₂, and VWF, but only Akt1−/− platelets show significant defects in response to collagen, indicating differences among Akt isoforms. Akt3−/− platelets exhibit a significant reduction in thrombin-induced phosphorylation of glycogen synthase kinase 3β (GSK-3β) at Ser⁹, which is known to inhibit GSK-3β function. Thus, Akt3 is important in inhibiting GSK-3β. Accordingly, treatment of Akt3−/− platelets with a GSK-3β inhibitor rescued the defect of Akt3−/− platelets in thrombin-induced aggregation, suggesting that negatively regulating GSK-3β may be a mechanism by which Akt3 promotes platelet activation. Importantly, Akt3−/− mice showed retardation in FeCl₃-induced carotid artery thrombosis in vivo. Thus, Akt3 plays an important and distinct role in platelet activation and in thrombosis.
Introduction

Platelets are critical for hemostasis, but under pathological conditions, are also important in thrombosis. Platelet activation is initiated at sites of vascular injury upon exposure to soluble agonists such as thrombin, ADP, and thromboxane A2, and adhesion to subendothelial matrix proteins, such as von willebrand factor and collagen. These adhesive proteins and agonists stimulate an intracellular signal transduction cascade leading to transformation of the major platelet adhesion receptor, integrin \( \alpha_{\text{IIb}} \beta_3 \) from its resting to active state (inside-out signaling), which allows the integrin to bind fibrinogen, and therefore mediate platelet aggregation. Activated platelets secrete proaggregating factors and adhesive glycoproteins from granules, which further cause stabilization and amplification of aggregation, leading to thrombus formation. Ligand binding to the activated integrin \( \alpha_{\text{IIb}} \beta_3 \) also transmits “outside-in” signals, which are critically important in stable platelet adhesion, spreading, and clot retraction.

Elucidation of the signaling pathways regulating platelet activation is essential for the identification of novel anti-thrombotic targets for the prevention of thrombosis, a major cause of heart attack and stroke. It is established that phosphoinositide (PI) 3-kinases (PI3K) play important roles in platelet activation. Akt (also known as Protein Kinase B or PKB), the most well known effector of PI3K, is activated downstream of PI3K during platelet activation. Akt is a family of serine/threonine kinases with three isoforms: Akt1, Akt2, and Akt3 (See reviews). Akt isoforms are 80% homologous in their protein sequences. However, knockout mouse models of Akt isoforms have revealed...
distinct phenotypes, suggesting the possibility of unique functional roles of Akt isoforms or differences in expression levels of Akt isoforms in specific tissues.\textsuperscript{17-19}

It has been demonstrated that platelets express Akt1 and Akt2.\textsuperscript{20} Knockout of Akt1 or Akt2 in mice results in similar defects in platelet activation induced by thrombin, VWF, and TXA\textsubscript{2}, but only Akt1\textsuperscript{+/−} platelets showed significant defects in collagen-induced platelet activation. These observations suggest that Akt1 and Akt2 are both important in platelet activation, but may have different roles.\textsuperscript{21-24} It has been reported that Akt3 was not detectable in platelets.\textsuperscript{20} However, in our study, we have obtained evidence that Akt3 is not only present in platelets, but also is a major Akt isoform expressed both in human and mouse platelets. Akt3 knockout mouse platelets selectively exhibit impaired platelet aggregation and secretion in response to stimulation by thrombin and TXA\textsubscript{2} receptors, but not collagen or VWF, which is different from either Akt1 or Akt2. Importantly, Akt3 knockout mice exhibit impaired thrombus formation \textit{in vivo} and \textit{in vitro} compared to wild type mice. Thus, Akt3 plays an important and distinct role in platelet activation and thrombosis.

\textbf{Methods}

\textbf{Animals}

The generation of Akt3 knockout mice has been previously described.\textsuperscript{19} Akt3\textsuperscript{+/−} mice are on a mixed 129R1/C57BL background. Wild type control mice and Akt3\textsuperscript{+/−} mice used in this study were 8-15 week-old littermates generated from heterozygous breeding. Animal
usage and protocol were 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 anti-coagulated with 1-seventh volume of acid-citrate dextrose (ACD) as 
previously described.\(^9\) 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 anti-coagulated with ACD as previously described.\(^{25}\) 
Blood from 5 to 6 mice of same genotype was pooled and platelets were isolated by 
differential centrifugation of whole blood with 0.1 \(\mu \text{g/mL}\) prostaglandin E\(_1\) and 1 U/mL 
apyrase (Sigma-Aldrich, St. Louis, MO). Platelets were washed twice with CGS buffer 
(sodium chloride 0.12 M, trisodium citrate 0.0129 M, D-glucose 0.03 M, pH 6.5), 
resuspended in modified Tyrode’s buffer and allowed to rest for at least 1 hour at room 
temperature before use.\(^{26}\) For some experiments, platelets were washed and resuspended 
according to Liu \textit{et al.}\(^{27}\)

**RT-PCR from platelet cDNA**

RNA was isolated from human, Akt3\(^{+/+}\) or Akt3\(^{-/-}\) mouse platelets (3x10\(^8\)) using Trizol 
Reagent (Invitrogen, Carlsbad, CA). Total RNA was reverse transcribed using 
Thermoscript RT-PCR kit (Invitrogen). Akt3 cDNA was amplified over 35 cycles with a 
forward 5’ ATG AAT TGT AGC CCA GCC TCA CAG ATT3’ and reverse 5’ CAT GCC
GTC GTC GTC ATA CTT TTC3’ primer for mouse Akt3; and with a forward 5’GAT GCC TCT ACA ACC CAT CAT3’ and reverse 5’GTC CAT GCA GTC CAT ACC ATC CT3’ for human Akt3. PCR products were separated on a 1% agarose gel containing ethidium bromide and visualized under UV lamp. Control PCR reaction was performed using the same cDNA preparations using primers specific for GAPDH. To exclude the possibility of contamination from leukocytes, RNA template was isolated from washed leukocytes and RT-PCR was performed using identical primers and PCR conditions as described for platelets. Isolation of leukocytes was performed as previously described.28

**Immunoblot detection**

Washed human platelets (1x10^9/mL) resuspended in Tyrode’s buffer were solubilized with an equal amount of solubilization buffer 1% Triton X-100, 150 mM NaCl, 50mM Tris, containing 10 mM EGTA, 0.2 mM E64, 1 mM phenylmethylsulfonyl fluoride, and 1 unit/ml aprotinin and incubated on ice for 20 min. After centrifugation at 13,000g for 20 min at 4°C, the lysates (300 μl) were preincubated with anti-Akt3 antibody or control IgG overnight. After incubation for 1 hour with Protein A/G conjugated Sepharose beads (Santa Cruz), beads were separated from the lysates by centrifugation. This procedure was repeated once, and then the immunoabsorbed platelet lysates were analyzed by SDS-PAGE and immunoblotted with an anti-Akt3 and anti-total Akt antibody. Experiment was repeated 4 times. Quantitation was performed using NIH Image J and paired t-test was used for statistical analysis.
Washed mouse platelets (3x10^8/mL) were incubated with or without thrombin in a platelet aggregometer at 37°C with stirring for various lengths of time. The reaction was stopped by addition of equal volume of sample buffer containing 2% SDS, 0.1M Tris, 2% glycerol, 2 mM PMSF, 2 mM Na3VO4, 2 mM NaF, and Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 4-15% polyacrylamide gel and then transferred to polyvinylidene difluoride membranes. The membranes were immunoblotted with an anti-Akt3 rabbit monoclonal antibody, anti-GSK-3β, anti-phospho-GSK-3β, anti-phospho Akt Thr308, anti-phospho Akt Ser473, anti-Akt1, anti-Akt2 (Cell Signaling Technology, Beverly, MA), and total Akt (recognizing Akt1, Akt2 and Akt3)(Santa Cruz Biotechnology, Inc).

**Platelet aggregation and secretion**

Platelet aggregation and secretion was measured in a turbidometric platelet aggregometer (Chronolog, Havertown, PA) at 37°C with stirring (1000 rpm). Washed platelets (3x10^9/mL) in modified Tyrode’s buffer were stimulated with thrombin (Enzyme Research Laboratories, South Bend, IN), collagen (Chronolog), U46619 (Calbiochem, La Jolla, CA), and vWF and botrocetin (kindly provided by Dr. Michael C. Berndt). Experiments were repeated at least three times. Platelet secretion was monitored in parallel with platelet aggregation as ATP release in a platelet lumiaggregometer (Chronolog) with the addition of luciferin/luciferase reagent (Chronolog) to the platelet suspension. Quantitation was performed using the ATP standard. To test the effects of GSK-3β inhibitor SB216763 (Sigma), SB216763 or dimethylsulfoxide (DMSO) was pre-
incubated with platelets at 37°C for 2 minutes before addition of agonist. Aspirin (Sigma) was dissolved as 40 mM stock solution in 0.2 M HEPES, 0.15 M NaCl, pH 7.8 (final pH 7.2) before added to platelets at 1 mM final concentration.

**Platelet adhesion under shear stress**

Glass slides were coated with collagen (50 μg/mL) in water with acetic acid added to pH 3.0 overnight. Slides were washed with PBS and blocked with 5% BSA in PBS for 1 hour and washed again with PBS. Washed mouse platelets (200μL of 3x10^8/mL) were loaded onto the slides. A cone and plate rheometer (Rheostress 1, Thermo-HAAKE, Paramus, NY) was used to introduce shear stress (800 s⁻¹) to the platelets. Mepacrine (10 μM; Sigma), a fluorescent dye was added to the platelets before applying shear stress to the platelets for 5 minutes. Slides were rinsed in a container with 200 mL PBS 3 times to wash out non-stably adherent platelets. Slides were viewed with a Leica DMI RB fluorescence microscope (Leica Microsystems) using an N PLAN L lens at 40x/0.55 NA objective with 1.5X magnification.

**In vivo thrombosis**

8-10 week old mice were anesthetized with isoflurane. The right carotid artery was isolated from surrounding tissues. A MA-0.5SB nanoprobe (Transonic Systems, Ithaca, NY) was hooked to the artery and blood flow was monitored with a TS420 flowmeter (Transonic Systems). After stabilization, 1.2 μL of 5% FeCl₃ (Sigma) was applied to a filter paper disc (2 mM diameter) that was placed on top of the artery for 2 minutes. After removing the filter paper, blood flow was monitored continuously until 5
minutes after occlusion. Time to occlusion was calculated as a difference in time between the removal of the filter paper and stable occlusion (no blood flow for 2 minutes). Statistical analysis was performed using the Mann-Whitney test for the evaluation of differences in median occlusion time.

Results

Akt3 is a significant Akt isoform expressed in platelets.

Important roles for Akt1 and Akt2 in platelets have been demonstrated by several groups. Akt3 was not detected in platelets in some previous studies. However, in our RT-PCR screen, a fragment of Akt3 mRNA was amplified using cDNA from purified platelets and oligonucleotide primers annealing to an Akt3-specific DNA sequence, and appeared to be abundant in human platelets (Fig. 1A) and wild type mouse platelets, but was not detected in Akt3−/− mouse platelets (Fig. 1B). It is unlikely that the detected Akt3 mRNA was from contaminating leukocytes in the platelet preparation, because Akt3 was not detected when RNA from the same numbers of leukocytes as that contaminating platelet samples was used as a template (Fig. 1B). Consistent with the expression of Akt3 mRNA in platelets, we also detected Akt3 protein in human and wild type mouse platelets, but not in Akt3−/− platelets by western blot with an Akt3-specific monoclonal antibody (Fig. 1C). These data indicate that Akt3 is indeed expressed in human and mouse platelets.

To determine the relative amount of Akt3 expressed in human platelets, washed human platelets were solubilized and the lysates were immunoprecipitated with an anti-Akt3
monoclonal antibody to deplete the Akt3 protein (Fig 1D, 1E). As shown in Fig. 2, this antibody is specific for Akt3 and does not cross react with Akt1 or Akt2, because no reactions were observed in Akt3−/− platelets, in which Akt1 and Akt2 are expressed normally. The levels of total Akt (all isoforms) or Akt3 that remained in platelet lysates were then determined by immunoblotting. Immunoabsorption with anti-Akt3 antibody, but not control IgG, depleted Akt3 from platelet lysates (Fig. 1D), and resulted in an approximate 35% reduction in total Akt; therefore, Akt3 constitutes approximately 35% of total Akt in human platelets (Fig. 1E). To assess the relative amount of Akt3 present in mouse platelets, total Akt and phosphorylated Akt levels in wild type and Akt3−/− platelets were measured by Western blot analysis using an antibody that recognizes all three isoforms and antibodies that recognize Thr308 and Ser473 phosphorylation sites of Akt, which are conserved among all Akt isoforms. Compared to wild type platelets, Akt3−/− platelets exhibit a ~70% reduction in total Akt (Fig. 2A, 2B). In wild type platelets, thrombin stimulates a time-dependent increase in phosphorylated Akt (at Thr308 and Ser473), which was also reduced by ~70% in Akt3−/− platelets (Fig. 2A, 2C and 2D). To exclude the possibility that Akt3 knockout caused dramatic changes in the expression levels of other Akt isoforms, we also analyzed Akt1 and Akt2 expression levels in wild type and Akt3−/− mouse platelets using isoform-specific antibodies against Akt1 or Akt2. No significant difference in Akt1 or Akt2 expression was observed in Akt3−/− mouse platelets compared to wild type (Fig. 2E), indicating that the loss of total Akt protein observed in Akt3−/− mouse platelets is not due to loss of Akt1 or Akt2 proteins. Thus, our data indicates that Akt3 is expressed in a significant amount in platelets, and activated by platelet agonists.
The role of Akt3 in platelet secretion and aggregation

Akt3+/− mice were used to investigate the role of Akt3 in platelet activation. Akt3+/− mice exhibit no obvious abnormality in major hematological parameters such as blood cell counts and hemoglobin levels. The size and counts of platelets in Akt3+/− mice are similar to that of wild type mice. Akt3+/− platelets showed a partial reduction and reversal in platelet aggregation induced by a low dose of thrombin (Fig. 3A), which is characteristic of a defect in platelet granule secretion. Platelet granule secretion is inhibited at this thrombin concentration (Fig. 3B). Correspondingly, the defects in aggregation and secretion in Akt3 knockout platelets were also observed at low concentrations of PAR4 agonist peptide, AYPGKF (Fig. 3C, 3D). At higher thrombin concentrations, the defect in platelet aggregation was overcome (Fig. 3A). However, the granule secretion in Akt3−/− platelets is still reduced compared to wild type platelets, indicating that the primary defect is in granule secretion (Fig. 3B). Indeed, supplementing with a low concentration of granule content ADP (1 μM), insufficient to induce aggregation on its own, reversed the inhibitory effect of Akt3 deficiency on thrombin-stimulated platelet aggregation (Fig. 3E). Thus, Akt3 plays an important stimulatory role in mediating platelet secretion, and the secretion-dependent second wave of platelet aggregation induced by thrombin. Akt3−/− platelets also show a partial decrease in granule secretion induced by thromboxane A2 (TXA2) analog, U46619 (Fig. 4A), and a delay in U46619-induced platelet aggregation (Fig. 4A). However, the defects of Akt3−/− platelets in platelet secretion and aggregation induced by thrombin appear to be independent of TXA2 signaling pathway because Akt3−/− platelets showed decreased thrombin-induced platelet aggregation compared to wild
type platelets in the presence of a high concentration of aspirin. Also, at the thrombin concentration in which aspirin reduced aggregation of wild type platelets, Akt3−/− platelet aggregation was further reduced by aspirin (Supplemental Fig. 1). The roles of Akt3 appear additive with that of Akt1/Akt2 because when SH-6, an isoform-nonselective inhibitor, is added to Akt3−/− platelets, platelet aggregation is further inhibited compared to Akt3−/− platelets alone (Fig. 3F). Thus, different isoforms of Akt all play important roles in thrombin- and TXA2-induced platelet granule secretion and the secretion-dependent second wave of platelet aggregation. In fact, the defects of platelet aggregation stimulated with thrombin in Akt1−/−, Akt2−/−, and Akt3−/− platelets appear very similar in direct comparison (Supplemental Fig. 3A), all showing a defective second wave and stability of aggregation. However, Akt3−/− platelets do not show significant defects in platelet aggregation and secretion induced by collagen (Fig. 4B), ADP (Fig. 4C), and VWF/botrocetin (Fig. 4D) even at very low concentrations. This selectivity is distinct from Akt1−/− platelets, which are defective in platelet activation induced by all these agonists, and also different from Akt2−/− platelets, which are defective in platelet activation induced by VWF, thrombin, and TXA2. Of the three isoforms, only Akt1 is required in platelet aggregation induced by low dose collagen, while neither Akt2 nor Akt3 knockouts showed significant effects on collagen-induced platelet activation (also see Supplemental Fig. 3B, 4B). Thus, our data indicate that Akt3 plays a stimulatory role selectively in the G protein-coupled thrombin receptor and the TXA2 receptor signaling pathways, which is distinct from Akt1 and Akt2. The difference in the roles of the 3 Akt isoforms in different platelet activation pathways suggests the different
mechanisms of action of Akt1, Akt2, and Akt3 in stimulating platelet activation. Nevertheless, there may also be overlapping functions of different Akt isoforms.

**The role of Akt3 in mediating GSK-3β phosphorylation**

Akt has numerous substrates. We have previously shown that the role of Akt1 in stimulating platelet secretion and aggregation is mainly mediated by the NO/cGMP signaling pathway. Our data also suggest that the role of Akt2 in GPIb-IX-dependent platelet activation is also mainly mediated via the cGMP pathway. The roles of Akt1 in the NO/cGMP pathway is consistent with the data that Akt1−/− platelets showed defects in platelet granule secretion induced by nearly all tested platelet agonists, as cGMP is elevated by all of these platelet agonists. However, Akt3−/− platelets selectively showed a defect in thrombin- and TXA2-induced platelet secretion and aggregation, suggesting a possibly different downstream effector. It has been established that phosphorylation of GSK-3β at Ser9 by Akt negatively regulates GSK-3β function. Interestingly, previous studies showed that GSK-3β plays a negative regulatory role selectively in thrombin-induced platelet aggregation, but plays a stimulatory role in collagen-induced platelet aggregation. Thus, we investigated the possibility whether GSK-3β is a downstream effector of Akt3. Indeed, thrombin induced a significant increase in the phosphorylation levels of GSK-3β in wild type platelets, which was partially, but significantly inhibited in Akt3 knockout platelets (Fig. 5A, 5B). The role of Akt3 in phosphorylation of GSK-3β is not limited to the thrombin pathway. Phosphorylation of GSK-3β induced by other agonists such as collagen and ADP was also partially inhibited in Akt3−/− platelets (Supplemental Fig. 2). In contrast to the partial
inhibition of GSK-3β phosphorylation in Akt3−/− platelets, treatment of platelets with the pan Akt inhibitor SH6 completely inhibited low-dose thrombin stimulated phosphorylation of GSK-3β at high SH6 concentrations (Fig. 5C), suggesting the involvement of other Akt isoforms in addition to Akt3. Previously, it has been shown that platelets lacking 3 alleles of Akt (Akt1+/−/Akt2−/−) exhibit a significant decrease in GSK-3β phosphorylation in PAR4 agonist peptide stimulated platelets compared to WT mouse platelets. Together, these data indicate that Akt3 plays an important role in mediating GSK-3β phosphorylation, but Akt isoforms other than Akt3 are also important in agonist-stimulated GSK-3β phosphorylation.

To further investigate whether the role of Akt3 in GSK-3β phosphorylation explains the selective role of Akt3 in thrombin-induced platelet aggregation, we tested the effects of a GSK-3β selective inhibitor, SB216763, on platelet aggregation induced by thrombin and collagen in wild type and Akt3−/− platelets. Consistent with a previous report, SB216763 enhanced platelet aggregation and secretion induced by subthreshold concentrations of thrombin (Fig. 6B). At higher concentrations of thrombin when platelet aggregation in normal platelets is already near maximal, there was no obvious effect of SB216763 on platelet aggregation (not shown). As expected, Akt3−/− platelets showed a decrease in platelet aggregation in response to low dose thrombin compared to wild type platelets. In contrast, Akt3−/− platelets treated with SB216763 (10 μM) totally rescued the defect of Akt3−/− platelets in thrombin-induced platelet aggregation (Fig. 6A). Because we have shown that Akt3 is important in mediating phosphorylation of GSK-3β, which inhibits GSK-3β function, and that inhibition of GSK-3β function enhances thrombin-induced aggregation.
platelet aggregation, the rescue of the aggregation defect in Akt3−/− platelets by GSK-3β inhibitor suggests that Akt3-mediated regulation of GSK-3β function is likely to be sufficient for the role of Akt3 in promoting thrombin-induced platelet aggregation. In contrast to thrombin-induced platelet aggregation, collagen-induced platelet aggregation and secretion was significantly inhibited by SB216763 (Fig. 6C), suggesting that GSK-3β does not promote platelet activation induced by the GPVI pathway. An inhibitory effect of SB216763 on platelet aggregation was also seen when ADP was used as an agonist (Fig. 6D). These data suggest that GSK-3β positively regulates GPVI and ADP receptor signaling pathways. However, Akt3 knockout did not enhance platelet aggregation induced by collagen and ADP (Fig 4), despite of a role of Akt3 in GSK-3β phosphorylation (Supplemental Fig 2), and SB216763 inhibited ADP-induced platelet aggregation in Akt3−/− platelets as in wild type platelets (Fig 6D). These data suggest either that the partial phosphorylation of GSK-3β by other Akt isoforms are sufficient to mediate its regulation in collagen and ADP signaling pathways or an uncharacterized stimulatory role of Akt3 in these pathways masked the functional effect of Akt3 knockout on GSK-3β phosphorylation. Together, our data indicate that the selective role for Akt3 in promoting thrombin-induced platelet aggregation may be mainly mediated by the Akt3-dependent phosphorylation of GSK-3β and consequent inhibition of the negative regulatory signal of GSK-3β, although we do not exclude that other possible downstream effectors of Akt3 may also contribute to its role in platelet activation.

The role of Akt3 in platelet adhesion and thrombus formation under flow conditions

*in vitro*
The important roles of different platelet activation pathways are to facilitate platelet adhesion and thrombus formation on exposed collagen surfaces of damaged blood vessel walls. To investigate whether the role of Akt3 in selective platelet activation pathways are relevant to thrombus formation on collagen surfaces under flow, we employed a cone and plate rheometer to introduce flow with controlled shear rates to platelets on a collagen coated surface. A shear rate of 800 s\(^{-1}\) was used to mimic the physiological shear rates of the arteries and arterioles. Washed platelets from both the wild type or Akt3\(^{-/-}\) mice stably adhered to the collagen-coated surface under this flow condition, suggesting that Akt3\(^{-/-}\) platelets are not defective in adhesion to collagen. However, in contrast to wild type platelets that formed large thrombi on the collagen coated surface, Akt3\(^{-/-}\) platelets only formed smaller aggregates (Fig. 7A, B), suggesting that Akt3\(^{-/-}\) platelets are deficient in recruiting platelets into thrombi under high shear rate conditions.

**Akt3 promotes in vivo thrombosis**

In order to determine the *in vivo* physiological relevance of the role of Akt3 in promoting platelet secretion and aggregation, we compared the *in vivo* thrombosis of wild type and Akt3\(^{-/-}\) mice using the FeCl\(_3\)-induced carotid artery thrombosis model. The time to the formation of stable occlusive thrombus in the carotid artery is significantly prolonged in Akt3\(^{-/-}\) mice compared with wild type control mice (p=0.0007, n=10 for Akt3\(^{-/-}\), n=10 for WT) (Fig. 7C). However, FeCl\(_3\)-induced carotid artery thrombosis still occurred in Akt3\(^{-/-}\) mice after a delay. This significant, but relatively mild anti-thrombotic effect of Akt3 knockout is consistent with its *in vitro* effect on thrombin and TXA\(_2\) induced platelet
secretion and aggregation. These data suggest that Akt3 plays an important regulatory role in thrombus formation in vivo.

Discussion

In this study, we show that Akt3 is a significant Akt isoform expressed in platelets and plays an important role in platelet activation that is different from that of Akt1 and Akt2. We show that the role of Akt3 in platelets is likely mediated by phosphorylating and thus, negatively regulating GSK-3β. Furthermore, we demonstrate an important role for Akt3 in promoting stable thrombus formation in vivo.

It is well established that the PI3K signaling pathway has a critical function in platelet activation.7,9,10 The Akt family of protein kinases is an important downstream effector of PI3K signaling. Recent studies have shown that Akt1 and Akt2 play important stimulatory roles in platelet activation induced by low concentrations of platelet agonists.21-24 The role of Akt3 in platelets has never been studied, possibly due to a previous report that Akt3 was not detectable in platelets.20 However, our data clearly show that Akt3 is expressed in platelets in a substantial amount. Furthermore, our data indicate that Akt3-/- platelets have defects in thrombin (and TXA2)-induced platelet granule secretion and aggregation in vitro, and in thrombus formation in vivo, which are consistent with previous data that G protein-coupled thrombin receptor and TXA2 receptor pathways are important in thrombosis in vivo. Thus, we conclude that Akt3 plays important roles in platelet function and thrombosis. Together with previous findings of important roles of Akt1 and Akt2 in platelets, and additive roles of Akt3 and other Akt isoforms, our data
further suggest that all three isoforms of Akt are important in promoting platelet granule secretion and thrombus formation, regardless of their relative quantities.

The role of Akt3 in promoting platelet activation appears to be selective for some of the platelet activation pathways, but is not involved in a general signaling pathway. Akt3−/− platelets showed significant defects in platelet activation induced by thrombin receptor agonists and (to a lesser degree) TXA2-mediated platelet secretion and aggregation, but had no defect in platelet secretion and aggregation induced by collagen, ADP and VWF/botrocetin. The defect of Akt3−/− platelets selectively in certain platelet activation pathways is clearly different from Akt1−/− platelets, which have been shown to be defective in platelet secretion and aggregation induced by all tested agonists including VWF, collagen, and ADP,21-24 and is also different from Akt2−/− platelets which is involved in VWF/GPIb-mediated platelet activation, in addition to thrombin and TXA2 pathway. Thus, the three isoforms of Akt play different roles in different platelet activation signaling pathways. Most of our current knowledge on the roles of Akt family of kinases in physiology and pathology were obtained with the prototype Akt isoform, Akt1 and to a degree, Akt2. Akt3 has been the least characterized, perhaps because Akt3 was thought to be a “redundant” Akt isoform. The functional redundancy between different Akt isoforms is evident, since more dramatic defects and early death only occur in mice with the genes of at least two Akt isoforms deleted. However, deletion of different individual Akt isoforms results in different phenotypes, suggesting that different Akt isoforms may also play distinct roles.19 Although, it is also possible that the distinct phenotype results from different tissue distribution patterns or localization of Akt
isoforms. Our studies provide novel evidence of distinct roles of different Akt isoforms in
the same cell.

Akt family of protein kinases has many substrates, and plays multiple roles in many
aspects of life and in many types of cells. For example, Akt1 has been shown to
phosphorylate and activate endothelial nitric oxide synthase (eNOS), and thus stimulate
nitric oxide (NO)-cGMP signaling pathway in many types of cells including platelets. Akt2
has been shown to be important in GPIb-IX-mediated cGMP elevation. However, how Akt2 plays a role in platelet activation remains unclear. The data presented in this study suggest that Akt3 mediates platelet activation mainly by negative regulation of another Akt substrate, GSK-3β.

It has been established that phosphorylation of GSK-3β at Ser9 by Akt family of kinases negatively regulates GSK-3β function. The role of GSK-3β in platelet activation has been examined by several groups. Some studies showed that pharmacological inhibition of GSK-3β reduced platelet aggregation induced by collagen, but another group showed that both pharmacological GSK-3β inhibitors and partial reduction of GSK-3β in heterozygous GSK-3β knockout platelets enhanced platelet aggregation induced by thrombin receptor agonists. In our studies, GSK-3β selective inhibitor SB216763 inhibited collagen- and ADP-induced platelet activation, but enhanced platelet aggregation induced by a subthreshold concentration of thrombin, suggesting that GSK-
3β plays differential roles in different platelet activation pathways, which explains previous controversies. The differential roles of GSK-3β in different platelet activation pathways are consistent with the selective role of Akt3 in different platelet activation pathways. Indeed, we observed a significant reduction in GSK-3β phosphorylation in Akt3−/− platelets stimulated with thrombin, indicating that Akt3 is an important kinase responsible for GSK-3β Ser9 phosphorylation. Furthermore, treatment of Akt3−/− platelets with GSK-3β inhibitor SB216763 completely corrected the defect in thrombin-induced platelet aggregation in Akt3−/− platelets. These data suggest that Akt3 is likely to play a stimulatory role in low dose thrombin-induced platelet activation by inactivating the negative regulatory role of GSK-3β. It is important to note that GSK-3β is regulated not only by Akt3, but also by other Akt isoforms, which may also contribute to its function. However, clearly different functional phenotypes between three different Akt isoforms suggest that other downstream effectors, such as the NO/cGMP pathway, may be important in the functional effects of these Akt isoforms. Also, we do not exclude the possibility that Akt3 may also regulate other unidentified substrates, which is interesting for further study. The mechanism by which GSK-3β exerts its effects is unclear at this stage. A recent study suggests a role for GSK-3β in the Wnt signaling pathway in platelets.37 Thus, more detailed studies to determine the roles of GSK-3β on platelet function downstream from Akt isoforms are warranted.

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Authorship

Kelly O’Brien performed a major part of experiments, analyzed data and wrote the paper.

Aleksandra Stojanovic-Terpo performed a part of the experiments and data analysis.

Nissim Hay was involved in research design, data analysis, discussions and provided Akt3−/− mice. Xiaoping Du designed the research, analyzed data and wrote the paper.

There are no conflicts of interest to disclose.

References


**Figure legends**

**Figure 1. Expression of Akt3 in platelets.** (A) Human platelet RNA was isolated from washed platelets (3 x10^8). RT-PCR was performed with primers specific for Akt3 or a housekeeping gene, GAPDH. (B) Mouse platelet RNA was isolated from 3 x10^8 platelets of wild type or Akt3⁻/⁻ platelets and RT-PCR was performed similarly. Leukocyte contamination of platelet preparation was 4 x 10^4/mL as determined using Hemavet blood cell analyzer. RNA was isolated from 4 x 10^4/mL of WT mouse leukocytes and was also analyzed by RT-PCR using Akt3 specific primers under the same conditions as for platelet preparations to verify that the Akt3 fragment was not from leukocyte contamination. (C) Washed human platelets, wild type and Akt3⁻/⁻ mouse platelets were solubilized and immunoblotted with a rabbit antibody specifically recognizing Akt3, and α-tubulin is used as loading control. (D) Washed human platelets were solubilized, and
immunoabsorbed with anti-Akt3 to remove Akt3 from lysates or with control rabbit IgG, and then immunoblotted with anti-Akt3 or an antibody recognizing all Akt isoforms (Total Akt). (E) Experiments in (D) were scanned and quantified using NIH Image J for uncalibrated optical density (mean ± SE, 4 experiments). The difference in % of total Akt between IgG and Akt3 immunoabsorbed lysates is significant (p<0.0125), as determined using paired t-test.

**Figure 2. Total and phosphorylated Akt in WT and Akt3−/− platelets.** (A and D) Washed Akt3−/− and WT mouse platelets were stimulated with thrombin (0.018 U/mL) for 1, 3, and 5 minutes, solubilized, and immunoblotted with antibodies directed against: (A) Akt3, total Akt (Akt1, Akt2 and Akt3), phosphorylated Ser473 of Akt, and GSK-3β (loading control); and (D) phosphorylated Thr308 of Akt, total Akt and α-tubulin (loading control). (B and C) Western blot results from each of three experiments as shown in (A) were scanned and quantitated using NIH Image J for uncalibrated optical density. The relative quantity of total Akt (B) and phosphorylated Ser473 of Akt (C) in wild type and Akt3−/− platelets are shown (mean ± SE). (E) Washed Akt3−/− and WT mouse platelets were solubilized and immunoblotted with antibodies directed against Akt1, Akt2, and Akt3 and α-tubulin (loading control).

**Figure 3. Stimulatory role of Akt3 in platelet aggregation and secretion in response to thrombin and PAR4 agonist peptide.** (A, B, C, D) Washed wild type (WT) and Akt3−/− platelets were stimulated with thrombin (A and B) or PAR4 agonist peptide AYPGKF (C and D). Platelet aggregation was monitored using a turbidometric
aggregometer at 37°C and 1,000 rpm stirring speed (A and C). Platelet secretion of ATP was recorded concomitantly in the presence of luciferin-luciferase agent (B and D). Experiments described in A, B, C, and D were repeated three times at a low dose of thrombin (0.02 U/mL) or AYPGKF (60 μM) with the results quantified as percentage of light transmission (mean ± SE) or as concentration of secreted ATP (mean ± SE). (E) A low concentration of ADP (1 μM), insufficient to induce aggregation on its own, reversed the inhibitory effect of Akt3−/− platelets on thrombin-stimulated aggregation. (F) Akt3−/− and WT mouse platelets were treated with vehicle DMSO or SH-6 (15 μM) for 2 minutes and aggregation was recorded as shown in (A).

**Figure 4. Responses of Akt3−/− platelets to other platelet agonists.** (A) Washed wild type (WT) and Akt3−/− platelets were stimulated with (A) U46619. Platelet aggregation was monitored using a turbidometric aggregometer at 37°C and 1,000 rpm stirring speed. Platelet secretion was recorded concomitantly in the presence of luciferin-luciferase agent. Platelet aggregation was also measured following stimulation with (B) collagen (For 0.8μg/mL, n=6, p=0.909), (C) ADP (For 2.5μM, n=4, p=0.1646) and (D) botrocetin and VWF (For Botrocetin 1.0μg/mL, n=3, p=0.837). Quantitation was performed using student’s t-test.

**Figure 5. Phosphorylation of GSK-3β.** (A) Washed Akt3−/− and WT mouse platelets were stimulated with thrombin (0.018 U/mL) for 1, 3, and 5 minutes, solubilized, and immunoblotted with antibodies against phosphorylated Ser9 of GSK-3β, and total GSK-3β (loading control). (B) Western blot results from each of three experiments as shown in
(A) were scanned and quantified using NIH Image J for uncalibrated optical density. The relative quantity of phospho-GSK-3β in thrombin stimulated Akt3−/− vs. that of wild type mouse platelets is expressed as the percentage of wild type (mean ± SE, 5 experiments). The difference in GSK-3β phosphorylation at resting, 1 min, 3 min, and 5 min time points are significant between WT and Akt3−/− (p<0.05), as determined using Student’s t test. (C) Washed WT mouse platelets were preincubated for 2 min with increasing doses of SH-6 or vehicle control DMSO and stimulated with thrombin (0.018 U/mL) for 3 minutes, solubilized, and immunoblotted with antibodies against phospho-Ser9 of GSK-3β, phospho-Ser473 of Akt, total Akt, and total GSK-3β (loading control).

**Figure 6. Reversal of the inhibitory effect of Akt3 knockout on platelet aggregation by GSK-3β inhibitor SB216763.** (A) Washed WT and Akt3−/− platelets were preincubated for 2 min with 10 μM of SB216763 or vehicle DMSO. These platelets were then stimulated with thrombin in a platelet aggregometer. (B) Washed WT mouse platelets were preincubated for 2 min with 10 μM of SB216763 or vehicle DMSO and stimulated with a subthreshold concentration of thrombin in a platelet aggregometer. Platelet secretion was recorded concomitantly with aggregation in the presence of luciferase agent, and ATP release was recorded. (C) Washed mouse platelets were preincubated for 2 min with 10 μM of SB216763 or vehicle DMSO and then stimulated with collagen. Platelet ATP secretion was measured concomitantly. (D) Washed WT mouse platelets or Akt3−/− platelets were preincubated with 10 μM of SB216763 or vehicle DMSO and then stimulated with ADP (5 μM) in the presence of fibrinogen (10 μg/mL) and platelet aggregation was recorded.
Figure 7. Akt3 knockout delays formation of stable thrombi. (A) Washed mouse platelets (200μL, 3x10^8/mL) were loaded onto slides coated with 50 μg/mL collagen and a cone and plate rheometer was used to introduce shear stress (800 s⁻¹) to the platelets. Mepacrine, a fluorescence dye was added to the platelets before applying shear stress for 5 minutes. Slides were rinsed in a container with PBS to wash out non-stably adherent platelets. Slides were viewed with a Leica DMI RB fluorescence microscope (Leica Microsystems). (B) Quantitation of (A) using t-test (p<0.001). (C) FeCl3- induced carotid artery injury was performed, and time to occlusive thrombosis was recorded as described under “Experimental Procedures.” The occlusion time of each mouse is shown as squares (Akt3⁻/⁻, n=10) and triangles (Akt3⁺/⁺, n=10). The bars represent the median occlusion time. Statistical analysis was performed using the Mann-Whitney test to evaluate the differences in median occlusion time (p= 0.0007).
Figure 1

A. Human Platelets

B. Mouse platelets

Leukocytes

WT

Akt3−/−

WT

B. Mouse platelets

Leukocytes

WT

Akt3−/−

WT

C. Human

Akt3−/−

WT

D. Immuno-absorption

IgG

Akt3

Akt3

Total Akt

E. Total Akt (% of control)

IgG

Akt3

*
Figure 6

A

Light Transmittance

Thrombin 0.02 U/mL

Akt3−/−

WT + SB216763

WT + SB216763

B

Light Transmittance

Thrombin 0.016 U/mL

WT + DMSO

WT + SB216763

C

Light Transmittance

Collagen 1 μg/mL

WT + SB216763

WT + DMSO

D

Light Transmittance

ADP 5μM

ATP Secretion (μM)

WT + SB216763

WT + DMSO

Akt3−/− + SB216763

Akt3−/− + DMSO
Figure 7

(A) Representative images of platelet aggregates in Akt3−/− and Akt3+/+ mice. (B) Bar graph showing the total area of platelets (pixels $10^4$/field) in Akt3−/− and Akt3+/+ mice. (C) Scatter plot showing the time to occlusion (sec) for Akt3−/− and Akt3+/+ mice.
An important role for Akt3 in platelet activation and thrombosis

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