Arhgef1 Plays a Vital Role in Platelet Function and Thrombogenesis

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Background—Platelets are the cellular mediators of hemostasis and thrombosis, and their function is regulated by a number of molecular mediators, such as small GTPases. These small GTPases are themselves regulated by guanine nucleotide exchange factors such as Arhgefs, several of which are found in platelets, including the highly expressed Arhgef1. However, the role of Arhgef1 in platelets has not yet been investigated.

Methods and Results—We employed mice with genetic deletion of Arhgef1 (ie, Arhgef1<sup>−/−</sup>) and investigated their platelet phenotype by employing a host of in vivo and in vitro platelet assays. Our results indicate that Arhgef1<sup>−/−</sup> mice had prolonged carotid artery occlusion and tail bleeding times. Moreover, platelets from these mice exhibited defective aggregation, dense and α granule secretion, αIIbβ3 integrin activation, clot retraction and spreading, in comparison to their wild-type littermates. Finally, we also found that the mechanism by which Arhgef1 regulates platelets is mediated in part by a defect in the activation of the RhoA–Rho-associated kinase axis, but not Rap1b.

Conclusions—Our data demonstrate, for the first time, that Arhgef1 plays a critical role in platelet function, in vitro and in vivo. (J Am Heart Assoc. 2019;8:e011712. DOI: 10.1161/JAHA.118.011712.)

Key Words: Arhgef1 • cardiovascular diseases • platelets • small GTPases • thrombosis

Platelets are the cellular mediators of hemostasis and thrombosis. Under normal conditions and upon injury to a blood vessel, platelets undergo drastic shape change, bind to adhesive protein substrates, and aggregate to form a plug, ultimately halting bleeding. Unfortunately, hyperactivity of platelets serves as a precursor to thrombotic diseases such as pulmonary embolism and myocardial infarction.

Platelet activation is regulated by a host of complex molecular events that modulate adhesion, aggregation, and thrombus formation, and include phosphorylation, as well as calcium-based and lipid-based signals. These events are coordinated by a number of molecular mediators such as small GTPases, which represent a family of low-molecular-weight (21–25 kDa) monomeric guanine nucleotide–binding proteins. These proteins work as intracellular “on-off” signaling switches; coordinating signal transduction and amplification. Several subfamilies of small GTPases have been identified in platelets and are cardinal regulators of the actin cytoskeleton dynamics, affecting platelet aggregation, secretion, spreading, and thrombus formation. They govern the diverse cellular processes through cycling between an inactive GDP-bound form and an active GTP-bound form through a process of GDP/GTP replacement. Importantly, the GDP/GTP cycling is highly regulated by a group of proteins/protein domains known as guanine nucleotide exchange factors (GEFs) such as Arhgefs.

While the Rho family of GEFs—a few of which have been identified in platelets—has been thoroughly studied in other cell systems, their role in platelets has not yet been fully understood. To this end, it has been reported that: (1) Arhgef3 is not very important for platelet function, at least in mice; (2) Arhgef10 regulates platelet activation in vitro (eg, aggregation) and in vivo (eg, hemostasis); and (3) Arhgef12 is “essential” for normal platelet function. While Arhgef1 is another member of this family that is highly expressed in platelets, virtually nothing is known regarding its direct role in platelet function. Given that RhoA is a crucial regulator of platelet responses mainly activated downstream of G<sub>12/13</sub> and G<sub>q</sub> coupled receptors, that these G-proteins are known to play a vital role in platelet function,
activates $G_{13}$\(^{36}\) we sought to examine the role of Arhgef1 in platelet function in vitro and in vivo.

**Materials and Methods**

**Reagents and Materials**

Thrombin, collagen, stir bars, and other disposables were from Chrono-Log Corporation, whereas U46619 was purchased from Abcam. Fluorescein isothiocyanate–conjugated anti–P-selectin, anti-Arhgef1, anti-actin, anti-RhoA, anti-pROCK, and anti-Rap1b antibodies were purchased from Cell Signaling Technology, Inc, whereas the anti-$\beta 3$ and anti-$G_{13}$ antibodies were from Sigma Aldrich. The JON/A antibody was from Emfret analytics. Fibrinogen was purchased from Sigma Aldrich. The RhoA and Rap1b Activation Assay Biochem Kits were from Cytoskeleton Inc. Other reagents were of analytical grade.

**Animals and Genotyping**

Arhgef1\(^{-/-}\) mice were obtained from the Mary Lyon Centre at MRC Harwell (www.har.mrc.ac.uk), Oxfordshire, United Kingdom, and genotyped using a polymerase chain reaction (PCR)–based method. PCR was performed using the following primers: Arhgef1-F (CCTTTGGGCTCTGACCTCTG), Arhgef1-R (AAGCCGTCAGTCCTCTGTCC), and CAS1 (TCTGTCATCGTTATGCGCC) with the following PCR condition: denaturation: 94°C for 7 minutes, amplification: 94°C for 1 minute, 58°C for 1 minute, 72°C for 90 seconds for 34 cycles, and finally extension at 72°C for 10 minutes. After completion of the PCR cycle, amplified DNA samples were run in 2% agarose gel and visualized in the gel documentation system. The expected products per genotype are as follows: wild-type (WT): 520 bp, Het: 362/520 bp, and mutant: 362 bp. Mice were housed in groups of 1 to 5 at 24°C, under 12/12 light/dark cycles, with access to water and food ad libitum.

**Methods**

All experiments involving animals were performed in compliance with the institutional guidelines and were approved by The University of Texas at El Paso Institutional Animal Care and Use Committee.

The authors declare that all supporting data are available within the article data. Requests for detailed analytic methods will be addressed by the corresponding author. For requests for the Arhgef1 deletion mice, these will be referred by the corresponding author to the Mary Lyon Centre at MRC Harwell (www.har.mrc.ac.uk), Oxfordshire, United Kingdom, and handled as per our Material Transfer Agreement.

**Immunoblotting**

Immunoblotting was performed as previously described. B briefly, and to confirm Arhgef1 deletion and that its deletion did not impact the expression levels of $\beta 3$ and $G_{13}$, platelet proteins were separated by SDS-PAGE and transferred to Immobilon-P PVDF membranes (Millipore Corp). They were then probed with anti-Arhgef1, anti-$\beta 3$, anti-$G_{13}$, or anti-actin primary antibodies and visualized with an appropriate alkaline phosphatase–coupled secondary antibody using enhanced chemiluminescent substrate (GE Healthcare). For the RhoA–Rho-associated kinase (ROCK) axis, washed platelets (Arhgef1\(^{-/-}\) and WT) were stimulated with thrombin (0.1 U/mL) for 3 minutes, and processed as described above. Membranes were then probed with anti-RhoA, anti-pROCKII\(S\)er1366, and anti-actin antibodies, and visualized with an appropriate horseradish peroxidase–coupled secondary antibody using enhanced chemiluminescence substrate (Thermo Scientific). Images were obtained with ChemiDoc MP Imaging System (Bio-Rad).

**Tail Bleeding Time Assay**

The tail bleeding time assay was performed as previously described. Briefly, mice were anesthetized and placed on a homeothermic blanket (37°C) before the tail was transected 5 mm from the tip using a sterile scalpel. After transection, the tail was immediately immersed in saline (37°C constant temperature), and the time to bleeding cessation was measured. Bleeding stoppage was not considered complete until bleeding had stopped for 1 minute. For statistical analysis purposes, if bleeding did not stop in 10 minutes, the experiment was halted to limit blood loss from the mice and 10 minutes was considered the cutoff bleeding time. The number of WT and knockout (KO) mice used was 8 each.
In Vivo Ferric Chloride Carotid Artery Injury–Induced Thrombosis Model

This assay was performed as previously described. Briefly, mice were anesthetized with Avertin (2.5%), and the left carotid artery was exposed and cleaned with normal saline (37°C) before baseline carotid artery blood flow was measured with Transonic Micro-Flow Probe (Transonic Systems Inc). After stabilizing blood flow, 7.5% ferric chloride was applied to a filter paper (1-mm diameter) that was immediately placed on top of the carotid artery for 3 minutes. Blood flow was continuously monitored until it stopped completely (stable occlusion). Data were recorded as the time to vessel occlusion and calculated as the difference in time between stable occlusion and removal of the filter paper (with ferric chloride). An occlusion time of 20 minutes was considered as the cutoff time for statistical analysis. The number of WT and KO mice used was 8 each.

Peripheral Blood Cell Counts

Peripheral blood cell counts were performed on whole blood obtained from both Arhgef1−/− and WT mice, using a HEMAVET 950FS Multispecies Hematology System from Erba Diagnostics.

Murine Platelet-Rich Plasma Preparation

The Arhgef1−/− and WT mice were anesthetized, and blood was collected from the heart. Coagulation was inhibited by 0.38% sodium citrate solution (Fisher Scientific). Blood was centrifuged (237g for 15 minutes) at room temperature (RT), and the platelet-rich plasma was then collected. Platelets were counted with the HEMAVET 950FS Multispecies Hematology System, and the counts were adjusted to 7×10⁷ platelets per mL before each experiment.

Washed Platelets Preparation

Mouse blood was collected as discussed above, mixed with phosphate-buffered saline, pH 7.4, and incubated with prostaglandin I₂ (10 ng/mL; 5 minutes), followed by centrifugation at 237g for 10 minutes at RT. The pellets were resuspended in HEPES/Tyrode buffer (20 mmol/L HEPES/potassium hydroxide, pH 6.5, 128 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L MgCl₂, 0.4 mmol/L NaH₂PO₄, 12 mmol/L NaHCO₃, 5 mmol/L D-glucose) supplemented with 1 mmol/L EGTA, 0.37 U/mL apyrase and 10 ng/mL prostaglandin I₂. Platelets were then washed and resuspended in HEPES/Tyrode (pH 7.4) without EGTA, apyrase, or prostaglandin I₂. Platelets were counted using the HEMAVET 950FS Multispecies Hematology System and adjusted to the indicated concentrations.

In Vitro Platelet Aggregation

Platelets from both Arhgef1−/− and WT mice were activated with the thromboxane receptor agonist U46619 (2.5 μmol/L), thrombin (0.05–0.1 U/mL), or collagen (5 μg/mL) and their aggregation response was measured by the turbidometric method using model 700 aggregometer (Chrono-Log Corporation). Each experiment was repeated 3 times using 3 separate groups, with blood pooled from 8 mice per group. Comparison is based on difference in maximal aggregation.

ATP Release

This assay was performed as previously described. Platelets were prepared as described above (250 μL; 7×10⁷/mL) before being placed into siliconized cuvettes and stirred for 5 minutes at 37°C. The luciferase substrate/luciferase mixture (12.5 μL, Chrono-Log) was then added, followed by the addition of the agonists U46619 (2.5 μmol/L), thrombin (0.05–0.1 U/mL), or collagen (5 μg/mL). Each experiment was repeated 3 times using 3 separate groups, with blood pooled from 8 mice per group. Comparison is based on difference in maximal secretion.

Flow Cytometric Analysis

Flow cytometry analysis was performed as previously described. Briefly, platelets (2×10⁷/mL) from Arhgef1−/− and WT mice were stimulated with U46619 (2.5 μmol/L), thrombin (0.1 U/mL), or collagen (5 μg/mL) for 5 minutes. Platelets were then fixed with 2% formaldehyde for 30 minutes at RT and incubated with fluorescein isothiocyanate–conjugated CD62P (P-selectin) or PE-conjugated rat anti-mouse integrin αIIbβ3 (active form) JON/A antibodies at RT for 30 minutes in the dark. The platelet (10⁵ platelets/100 μL) fluorescent intensities were measured using an Accuri C6 Flow Cytometer (BD Biosciences). Results were analyzed using C-Flow Plus (BD Biosciences). Each experiment was repeated 3 times using 3 separate groups, with blood pooled from 8 mice per group. Comparison is based on difference in mean fluorescent intensity.

Fibrin Clot Retraction Assay

Clot retraction assay was performed as previously described. Briefly, whole blood was collected and washed platelets were isolated as discussed above. CaCl₂ was added extemporaneously, at a final concentration of 1 mmol/L. The glass tubes that were used for aggregation (Chrono-Log...
Corporation) were employed for retraction assays. The washed platelets were resuspended at $1 \times 10^6$/mL in HEPES-Tyrode buffer (pH 7.4). Fibrinogen (500 µg/mL) was added in 0.5-mL platelets aliquots, and clot retraction was initiated by quickly adding thrombin (0.1 U/mL). The reaction was transferred to the glass tube and the reaction was set at RT. Pictures were taken at time intervals of 5 minutes up to half an hour using a digital camera. This experiment was repeated at least 3 times, with blood pooled from a group of 8 mice each time. Comparison is based on difference in clot size.

**Platelet Spreading**

The spreading of the Arhgef1$^{-/-}$ and WT platelets after stimulation with thrombin (0.1 U/mL) was examined as previously described. Briefly, sterile glass coverslips were coated with 0.2 µg/mL of fibrinogen for 30 minutes at RT. Washed platelets were placed onto these fibrinogen-coated coverslips for 5, 30, and 45 minutes before they were fixed with 3.7% (vol/vol) formaldehyde for 15 minutes and quenched with 50 mmol/L ammonium chloride. Cells were rinsed with PBS and incubated with tetramethylrhodamine-conjugated phalloidin (1 µg/mL) in 10% fetal bovine serum/PBS with 0.2% saponin. Coverslips were mounted and examined and imaged using a Leica DMI8 inverted widefield fluorescence microscope with integrated high-precision focus drive. Images were processed using LAS X Wizard imaging software. Objective lenses used were a ×63/×100 numeric aperture. Type A immersion oil (Fryer) was used for the ×63 objective. This experiment was repeated at least 3 times, with blood pooled from a group of 8 mice each time. Comparison is based on difference in level of spreading.

**Small-GTPase Pull-Down Assay**

The small GTPase pulldown assay was performed using the small GTPase pulldown kit according to the manufacturer’s instructions. Briefly, platelets (Arhgef1$^{-/-}$ and WT) were stimulated with thrombin (0.1 U/mL) for 0, 30, 60, 180, and 300 seconds and reactions were stopped with the lysis buffer. The lysates were cleared by centrifugation, and the supernatants were incubated with either rosetekin RBD for RhoA or GST-humanRalGDSRBD (788–885) for Rap1b for 1 hour at RT. Next, we collected the protein-bound bead by centrifugation at 3000g for 1 minute. The bead-bound complexes were washed 3 times with wash buffer. Finally, bead bound-complexes were eluted using 2 × sample buffer, separated by 12% SDS-PAGE, and transferred to Immobilon-P PVDF membranes (Millipore Corp). They were then probed with the indicated primary antibodies and visualized with an appropriate horseradish peroxidase-coupled secondary antibody using enhanced chemiluminescence substrate (Thermo Scientific). Images were obtained with a ChemiDoc MP Imaging System (Bio-Rad). This experiment was repeated 3 times using 3 separate groups, with blood pooled from 8 mice per group. Comparison is based on difference in RhoA and Rap1b activation.

**Statistical Analysis**

All experiments were performed 3 times using 3 separate groups, with blood pooled from 8 mice per group, as applicable. Data analysis was performed using GraphPad PRISM statistical software and presented as mean±SD. Mann–Whitney and t tests were used for the evaluation of differences in mean occlusion and bleeding times, whereas t test was used for analysis of the flow cytometry data. Notably, time-to-event analyses were used to confirm the bleeding and occlusion time analysis/results, given that the data were censored, and the findings confirm our original results. Significance was accepted at $P<0.05$.

**Results**

**Confirmation of Arhgef1 Deletion**

Given the important contribution of Arhgef1 in the activation of the G13-RhoA axis and G13 in platelet function and thrombogenesis, we sought to investigate the direct role Arhgef1 plays in platelet biology. In these studies, we employed mice with Arhgef1 deletion (Arhgef1$^{-/-}$). We first confirmed the successful deletion of the Arhgef1 gene (genotype) in our mice by PCR analysis of genomic DNA (Figure 1A), as well as by immunoblot (Figure 1B). Notably, the Arhgef1 KO mice appeared healthy and showed no physical differences in comparison to their WT littermates, and produced viable and healthy offspring, nor did the deletion of Arhgef1 impact the expression levels of G13 or the integrin β3 subunit (Figure 1C).

**Arhgef1 Deletion Alters Physiological Hemostasis and Thrombogenesis**

We subsequently investigated whether Arhgef1 regulates thrombogenesis by employing the ferric chloride carotid-injury model. The Arhgef1$^{-/-}$ mice were found to exhibit dramatically prolonged occlusion times, compared with their WT littermates mice (Figure 2A; median 217.5 and 803, whereas the interquartile range was 73.2 and 193 for WT and KO mice, respectively), indicating that these mice are protected against thrombus formation. In light of this finding, we next sought to examine the role of Arhgef1 in hemostasis by measuring the tail bleeding time. It was observed that the time needed for
cessation of bleeding following tail transection was significantly prolonged in the Arhgef1−/− mice, relative to the WT (Figure 2B; median 50 and 363, whereas the interquartile range was 52.5 and 341.5 for WT and KO mice, respectively). These data support the notion that Arhgef1 is essential for normal physiological hemostasis. Together, our findings suggest that Arhgef1 is a key player in in vivo platelet function.

Arhgef1 Deletion Does Not Affect Thrombopoiesis

To ensure that the observed defects in thrombogenesis and hemostasis are not caused by abnormalities in platelet number, the latter were analyzed in the Arhgef1−/− and WT mice in whole blood. Our data revealed no difference in platelet counts between the KO and WT mice (Table), nor were there any differences in the platelet mean volume or other blood cell counts (Table). These data support the notion that Arhgef1 is essential for normal physiological hemostasis. Together, our findings suggest that Arhgef1 is a key player in in vivo platelet function.

Arhgef1 Deletion Inhibits Agonist-Induced Platelet Aggregation

We first examined the role of Arhgef1 in platelet aggregation in response to activation of some of the major G-protein–coupled receptors (GPCRs), particularly those coupled to Gq and G13. It was observed that platelets from Arhgef1−/− mice exhibited lower aggregation, in comparison with their WT littermates, in response to the protease-activated receptor agonist thrombin (0.05−0.1 U/mL; Figure 3A and 3B; inset shows quantification of maximal aggregation), indicating a defective aggregation response. Furthermore, reduced aggregation was also found in response to the thromboxane A2 receptor agonist U46619 (2.5 μmol/L; Figure 3C; inset shows quantification of maximal aggregation). We next extended our studies to the agonist collagen, which acts through the non-GPCR glycoprotein VI; the latter is not known to directly associate with G13. Interestingly, we again observed impaired aggregation in the Arhgef1 deletion platelets, relative to those from the WT mice (Figure 3D; inset shows quantification of maximal aggregation).
Arhgef1 Regulates Platelet Function

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Arhgef1 Deletion Reduces Agonist-Induced Platelet Secretion

Given the important role of agonist-induced exocytosis (granules release) in amplifying initial platelet activation events,47,48 we studied the impact of Arhgef1 deletion on dense (ATP) and α-granules (P-selectin) secretion/release. Consistent with the aggregation data, the Arhgef1−/− mice had significantly lower thrombin-induced ATP secretion (0.05–0.1 U/mL; Figure 3E and 3F) and P-selectin expression (0.1 U/mL; Figure 4A), as well as reduced U46619-triggered ATP secretion (2.5 μmol/L; Figure 3G) and P-selectin expression (2.5 μmol/L; Figure 4B), compared with the WT mice. Similar results were observed with the non-GPCR agonist collagen (5 μg/mL; Figures 3H and 4C). These data provide evidence that Arhgef1 is essential for the platelet secretion response.

Arhgef1 Deletion Impairs Clot Retraction and Inhibits Platelet Spreading

Activation of integrin αIIbβ3 is required for clot retraction and platelet spreading through “outside in signaling,” which triggers an internal signaling cascade.14,50 In light of the critical function Arhgef1 is found to play in integrin αIIbβ3 activation, we investigated its role in clot retraction and platelet spreading. Our data show defective clot retraction and platelet spreading in the absence of Arhgef1 (Figure 5A and 5B), indicating that it participates in “outside in signaling.”

Collectively, our studies demonstrate that Arhgef1 is a key regulator of platelet activation in vitro and in vivo.

Arhgef1 Deletion Inhibits RhoA Activation and Phosphorylation of ROCK in Platelets But Has No Effect on Rap1b Activation

To elucidate the mechanism by which Arhgef1 regulates platelet function, we investigated the impact of its deletion on RhoA-ROCK axis. Our data show that Arhgef1 deletion inhibits RhoA activation in response to thrombin (0.1 U/mL) stimulation (Figure 6A). Notably, RhoA time course activation reveals that “maximal” activation occurs at 60 seconds, with the complete inactivation coinciding with 300 seconds in the WT platelets (Figure 6A). We further investigated the activation of the downstream/RhoA effector ROCK. Consistent with RhoA inhibition, phosphorylation of ROCK (Figure 6B) was also found to be reduced in thrombin (0.1 U/mL)-stimulated Arhgef1−/− platelets compared with the WT littermates. We next sought to determine whether the deficiency of Arhgef1 affects other small G-protein activation, namely Rap1b. Interestingly, we did not observe any detectable differences in Rap1b activation between the WT and Arhgef1−/− platelets (Figure 6C). These data suggest that Arhgef1 regulation of small G-protein activation is specific in nature.

Discussion

Given the importance of platelets in the genesis of cardiovascular disease, their activation mechanisms/signaling have been under intensive investigation for years, which revealed a complex array of pathways and communication at the molecular level.51 In this connection, several soluble agonists were found to activate platelets through binding to their receptors,52 mostly being GPCRs. As for their coupling profile, there is overlap in the G proteins to which these receptors are coupled to,52 with 4 different types identified in platelets including Gq, Gi3, Gi, and G12.53

Upon stimulation of their GPCRs, the G proteins’ α-subunits switch from the inactive GDP bound form, to the

**Table.** Peripheral Blood Cell Counts in WT and Arhgef1 KO Mice

|                | WT       | Arhgef1−/− | P Values |
|----------------|----------|------------|----------|
| Platelets      | 1133±57  | 1081±42    | 0.196    |
| MPV           | 4.56±2.66| 4.74±2.71  | 0.1484   |
| Red blood cells| 8.55±1.28| 8.65±1.39  | 0.176    |
| Lymphocytes    | 6.44±1.58| 6.75±1.41  | 0.148    |
| Monocytes      | 0.46±0.036| 0.41±0.027 | 0.184    |
| Granulocytes   | 2.49±0.23| 2.32±0.25  | 0.165    |

Blood was collected from the heart and counted as described in the Methods section. All counts are expressed as thousands per microliter, except for red blood cells, which are expressed as millions per microliter. Data are presented as mean±SD. KO indicates knockout; MPV, mean platelet volume; WT, wild-type.

Collectively, these findings indicate that Arhgef1 does indeed regulate platelet aggregation, including that which is not GPCR-mediated, and explains, at least in part, the in vivo phenotype.
GTP$^{54,55}$ version, thereby inducing internal signal transduction, such as Rho-GTPases. The Rho family of GTPases are molecular switches that control some of the most fundamental processes of cell biology, including regulation of gene expressions,$^{56}$ remodeling of the actin-based cytoskeleton,$^{57}$ and platelet function.$^{58,59}$ Moreover, $G_{13}$ and $G_q$, which are known to activate RhoA$^{34,60,61}$ and bind to GEFs first,$^{62,63}$ play a critical role in platelet function. While the role of $G_{13}$, 

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**Figure 3.** Arhgef1 deletion reduces platelets aggregation and dense granule secretion. A through D, Platelets from Arhgef1$^{-/-}$ and wild-type (WT) mice were stimulated with thrombin (A and B), U46619 (C), or collagen (D) before their aggregation response was monitored using an aggregometer (inset shows quantification of maximal aggregation). E through H, Platelets from Arhgef1$^{-/-}$ and WT mice were incubated with luciferase luciferin (12.5 μL) before being stimulated with thrombin (E and F), U46619 (G), or collagen (H). ATP release was detected as luminescence and measured by a lumiaggregometer. Each experiment was repeated at least 3 times, with blood pooled from a group of 8 mice each time (*$P<0.05$; **$P<0.01$).
Gq, and RhoA is well established in platelet function,\textsuperscript{4,34,35} little is known about the function of RhoGEFs. To this end, several RhoGEFs have been identified in platelets\textsuperscript{26} and found to play a critical role in their function, such as Arhgef3,\textsuperscript{30} Arhgef10,\textsuperscript{31} and Arhegf12.\textsuperscript{32} On the other hand, the role of another highly expressed GEF in platelets, namely Arhgef1, has not been elucidated. Thus in the present study, we aimed at characterizing the role of Arhgef1 in platelet function in vitro and in vivo by employing a genetic deletion mouse model.

The Arhgef1/RhoA signaling pathways have been recently shown to be involved in several physiological/pathological processes; for instance, Arhgef1 plays a role in arterial stiffness\textsuperscript{64} and atherosclerosis.\textsuperscript{65} In agreement with these reports, our findings reveal that Arhgef1 directly contributes to thrombogenesis, as its deletion prolonged vessel occlusion time (ie, protected against thrombus formation). This notion was further affirmed by the observation that Arhgef1 is also vital for hemostasis. Similarly, Arhgef10\textsuperscript{31} and Arhegf12\textsuperscript{32} were also found to regulate in vivo platelet function, unlike Arhgef3,\textsuperscript{30} whose deletion was not associated with a platelet phenotype in mice.\textsuperscript{30} These data provide evidence that Arhgefs play differential yet redundant roles in platelets. Notably, the platelet count and mean platelet volume were found to be normal in Arhgef1 KO mice. These findings indicate that the Arhgef1 phenotype observed—at least thus far—is not a consequence of an abnormality in thrombopoiesis. While this is consistent with what was observed with Arhgef10 and 12 KO mice,\textsuperscript{31,66} it is in contrast to the macro thrombocytopenia phenotype observed caused by RhoA deficiency and the increased mean platelet volume associated with Arhgef3 KO mice.\textsuperscript{30} These data further support the notion that differences do exist in the role of the various Arhgefs in platelet function and their downstream signaling.

We next sought to investigate the mechanism underlying the Arhgef1\textsuperscript{1−/−} defects in hemostasis and thrombogenesis. Given the importance of the G\textsubscript{13}RhoA pathway in platelet aggregation\textsuperscript{34,62} and secretion,\textsuperscript{33,67} we first examined the impact of Arhgef1 deletion on these functional responses.

![Figure 4. Arhgef1 deletion reduces α-granule secretion and integrin αIIbβ3 activation. Platelets from Arhgef1\textsuperscript{1−/−} and wild-type (WT) mice were washed before stimulation with thrombin, U46619, or collagen, and incubation with (A through C) fluorescein isothiocyanate–conjugated CD62P antibody (for α granules), or (D through F) fluorescein isothiocyanate–conjugated JON/A antibody, respectively. The fluorescent intensities were measured by flow cytometry. Average mean fluorescence intensities are shown. Each experiment was repeated at least 3 times, with blood pooled from a group of 8 mice each time.]

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Our data demonstrated that agonist-induced platelet aggregation, as well as secretion (i.e., dense and α granules), were reduced/defective in Arhgef1<sup>−/−</sup> platelets. These results are consistent with the defective dense and α granules secretion in the RhoA null platelets<sup>68</sup> downstream of G<sub>13</sub> and G<sub>q</sub>.

Figure 5. Arhgef1 deletion impairs clot retraction and platelet spreading. A, Whole blood was collected from both Arhgef1<sup>−/−</sup> and wild-type (WT) mice and washed platelets were isolated as discussed in the Methods section. Clot retraction was initiated by adding thrombin (0.1 U/mL) and photographed every 5 minutes for 30 minutes. B, Arhgef1<sup>−/−</sup> and WT platelets were allowed to adhere to fibronectin-coated coverslips for 5 minutes after stimulation with thrombin (0.1 U/mL). Tetramethylrhodamine-conjugated phalloidin in 10% fetal bovine serum/PBS with 0.2% saponin was used to stain for F-actin and imaged using Leica DMi8 inverted widefield fluorescence microscope with integrated high precision focus drive. Images were processed using LAS X Wizard imaging software. Data are representative of 3 independent experiments. Each experiment was repeated at least 3 times, with blood pooled from a group of 8 mice each time.
We also observed defects in integrin αIIbβ3 activation, clot retraction, and platelet spreading in the Arhgef1−/− platelets upon agonist treatment. These findings are not surprising given the role of Arhgef1 in platelet aggregation, and that of RhoA in integrin activation, “outside-in signaling,”69 and myosin light chain kinase phosphorylation68 (involved in integrin activation), as well as the notion that integrin αIIbβ3 “serves as a noncanonical Gα13-coupled receptor that provides a mechanism for dynamic regulation of RhoA.”70 While one cannot exclude secondary effects contributing to the in vivo defects, eg, as a result of the function of Arhgef1 in cell types other than platelets, the observed phenotype clearly derives, at least in part, from the defects in a host of platelet functional responses. Interestingly, Arhgef1 appeared to regulate non–GPCR-mediated platelet activation, namely that downstream of the collagen receptor glycoprotein VI; albeit the latter is not known to be associated with G13. These data indicate that Arhgef1 participates in noncanonical/non-GPCR signaling. It is noteworthy that Arhgef10 deletion platelets exhibited similar defects to those of Arhgef1,31 whereas there were no apparent defects in the Arhgef12 KO mice66 and the Arhgef3 were without a platelet function phenotype.30 Together, these findings provide further evidence that Arhgefs play differential roles in platelet function.

We also demonstrated that Arhgef1 plays an important role in activation of the RhoA signaling pathway. Thus, RhoA activation was impaired and so was the phosphorylation of downstream effectors (namely ROCK) in response to thrombin stimulation, which is consistent with the Arhgef10 deletion phenotype.31 In contrast, Arhgef12 was reported not to have any apparent role in RhoA activation in response to platelet stimulation.66 Interestingly, Arhgef1 was not found to regulate the activation of a separate small GTPase, namely Rap1b, which supports the notion that Arhgef1 regulates small GTPases in a specific fashion. Taken together, these findings further indicate that RhoGEFs play distinct roles in platelet function. Finally, while the RhoA pathway clearly plays a vital role in Arhgef1-mediated platelet activation, one cannot exclude the presence of an alternative pathway and/or a noncanonical role in platelet function (in light of the collagen phenotype), which will be the focus of future experiments.

Conclusions

Our data show that Arhgef1 regulates the RhoA-ROCK axis and is essential for normal platelet function, as well as for hemostasis and thrombogenesis. These findings may define Arhgef1 as a novel therapeutic target for managing thrombosis-based cardiovascular disease.

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Disclosures

None.

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