Receptor-interacting protein kinase 3 promotes platelet activation and thrombosis

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Previous studies have shown that receptor-interacting protein kinase 3 (RIP3) is involved in many important biological processes, including necroptosis, apoptosis, and inflammation. Here we show that RIP3 plays a critical role in regulating platelet functions and in vivo thrombosis and hemostasis. Tail bleeding times were significantly longer in *RIP3*-knockout (RIP3$^{-/-}$) mice compared with their wild-type (WT) littermates. In an in vivo model of arteriole thrombosis, mice lacking RIP3 exhibited prolonged occlusion times. WT mice repopulated with RIP3$^{-/-}$ bone marrow-derived cells had longer occlusion times than RIP3$^{-/-}$ mice repopulated with WT bone marrow-derived cells, suggesting a role for RIP3-deficient platelets in arterial thrombosis. Consistent with these findings, we observed that RIP3 was expressed in both human and mice platelets. Deletion of RIP3 in mouse platelets caused a marked defect in aggregation and attenuated dense granule secretion in response to low doses of thrombin or a thromboxane A_2 analog, U46619. Phosphorylation of Akt induced by U46619 or thrombin was diminished in RIP3$^{-/-}$ platelets. Moreover, RIP3 interacted with Gα_{13}. Platelet spreading on fibrinogen and clot retraction were impaired in the absence of RIP3. RIP3 inhibitor dose-dependently inhibited platelet aggregation in vitro and prevented arterial thrombus formation in vivo. These data demonstrate a role for RIP3 in promoting in vivo thrombosis and hemostasis by amplifying platelet activation. RIP3 may represent a novel promising therapeutic target for thrombotic diseases.

platelets | receptor-interacting protein kinase 3 | thrombin | thromboxane A2 | thrombosis

On vessel injury, platelets are recruited by soluble agonists, such as ADP, thrombin, and thromboxane A_2 (TXA_2), which interact with G protein-coupled receptors (GPCRs), leading to integrin inside-out activation and granule secretion (1–4). Ligand binding to integrin triggers outside-in signaling, initiating stable adhesion, spreading, clot retraction, and a second wave of secretion, which can result in an irreversible platelet plug and serious cardiovascular thrombotic events, including myocardial infarction and stroke (1, 5–7). Despite the well-established knowledge of the mechanisms of thrombus formation, which has contributed to the development of antithrombotic drugs, the signaling cascades leading to platelet activation are not completely understood.

The receptor-interacting protein kinase (RIP) family is a group of Ser/Thr protein kinases that share a highly homologous amino terminal kinase domain but have distinctly different carboxyl termini (8, 9). The family comprises seven members, designated RIP1–7 (8, 9). RIP3 has a carboxyl terminus that contains the RIP homotypic interaction motif, which mediates its interaction with other necroptosis-related proteins (10, 11). Downstream of death receptors, Toll-like receptors, or other sensors, RIP3 activation is tightly regulated by phosphorylation (12–15) or caspase-mediated cleavage (16, 17). Once activated, RIP3 phosphorylates its substrates, leading to programmed necrosis (13–15). Many phosphoproteins associated with the cell cycle, metabolism, or other diverse functions are regulated by RIP3 (18), suggesting that RIP3 might function beyond programmed necrosis. Indeed, the roles of RIP3 in embryonic development and various disease pathologies, such as inflammation and apoptosis, have been reported (8–10). A recent report indicated that RIP3-mediated necrotic death in areas of atherosclerotic plaques accelerates systematic inflammation and results in premature death of the animal, suggesting the possible involvement of RIP3 in cardiovascular diseases (19). The role of RIP3 in thrombosis and hemostasis has not been explored previously, however.

In this study, we demonstrate that RIP3 is expressed in both human and mouse platelets and promotes hemostasis and thrombus formation in vivo. We show that RIP3 selectively amplifies thrombin- and thromboxane A_2 analog (U46619)-induced platelet aggregation and dense granule secretion. We found that RIP3 interacts with Gα_{13}, and that Akt activation, spreading on fibrinogen, and clot retraction are defective in RIP3-deficient platelets. Moreover, RIP3 inhibitor dose-dependently inhibits platelet aggregation in vitro, and prevents thrombus formation in vivo. These results demonstrate a novel role for RIP3 in amplifying platelet activation and thrombus formation.

Results

RIP3$^{-/-}$ Mice Display Impaired Hemostasis and Thrombosis. To investigate the role of RIP3 in hemostasis in vivo, we examined tail bleeding times in RIP3$^{-/-}$ mice. The generation of RIP3$^{-/-}$ mice has been described previously (11). The RIP3$^{-/-}$ and wild-type

Significance

Receptor-interacting protein kinase 3 (RIP3) is involved in many important biological processes such as necroptosis, apoptosis, and inflammation. Here, using *RIP3*-knockout mice, we show that RIP3 is essential for in vivo thrombosis and hemostasis. Mice lacking RIP3 exhibited prolonged tail-bleeding times and reduced arterial thrombus formation. We demonstrate that RIP3, expressed in platelets and interacting with Gα_{13}, promotes platelet aggregation, secretion, spreading on fibrinogen, and clot retraction. RIP3 inhibitor dose-dependently inhibits platelet aggregation and prevents arterial thrombus formation. Our findings indicate a role for RIP3 in promoting in vivo thrombosis and hemostasis by amplifying platelet activation. RIP3 may represent a novel therapeutic target for thrombotic diseases.

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(WT) mice did not differ significantly in terms of RBCs, WBCs, or hemoglobin concentration (Table S1). RIP3$^{-/-}$ mice exhibited no tendency toward spontaneous bleeding or thrombotic events over the lifespan; however, their median tail bleeding time was significantly longer than that of WT littermates (410 s vs. 156 s; $P < 0.001$) (Fig. 1*A*). Moreover, in 44.4% of the RIP3$^{-/-}$ mice (vs. 16.7% of WT mice), bleeding persisted during a 10-min observation period. These data indicate that RIP3 plays an important role in primary hemostasis.

We investigated the role of RIP3 in arterial thrombosis in vivo using an $FeCl_3$-injured mouse mesenteric arteriole thrombosis model. Platelet thrombus formation was monitored in real time by fluorescence microscopy. Compared with their WT littermates, RIP3$^{-/-}$ mice exhibited delayed and diminished thrombus formation (Fig. 1*B*). Mean occlusion time was significantly longer in the RIP3$^{-/-}$ mice (19.9 ± 1.1 min vs. 13.5 ± 1.2 min; $P = 0.0012$) (Fig. 1*C*). These data demonstrate that RIP3 promotes arteriole thrombus formation in vivo.

RIP3 Is Expressed in Platelets and Responsible for Impaired Platelet Thrombus Formation in RIP3$^{-/-}$ Mice. Platelets play key roles in thrombosis and hemostasis. Our immunoblotting data indicate that RIP3 is expressed in both human and mouse platelets (Fig. 2*A*). As expected, RIP3 protein is ablated in the platelets from RIP3$^{-/-}$ mice, with WBCs as positive controls (20). To exclude a possibility that the defects in thrombosis and hemostasis of the RIP3$^{-/-}$ mice results from lack of RIP3 in vascular cells, such as endothelial cells, WT and RIP3$^{-/-}$ mice were repopulated with RIP3$^{-/-}$ or WT mouse bone marrow-derived cells, respectively, via bone marrow transplantation. The expression levels of RIP3 in platelets from RIP3$^{-/-}$ mice repopulated with WT mouse bone marrow-derived cells were similar to those in platelets from WT mice, whereas RIP3 expression in the platelets from WT mice repopulated with RIP3$^{-/-}$ bone marrow-derived cells was abolished (Fig. 2*B*). Occlusion times were significantly longer in WT mice repopulated with RIP3$^{-/-}$ bone marrow compared with RIP3$^{-/-}$ mice repopulated with WT bone marrow (18.4 ± 1.2 min in RIP3$^{-/-}$→WT mice vs. 12.5 ± 0.9 min in WT→RIP3$^{-/-}$ mice; $P = 0.0047$) (Fig. 2*C*). Moreover, occlusion times of WT→WT and WT→RIP3$^{-/-}$ mice were similar to those of WT mice, and occlusion times of RIP3$^{-/-}$→RIP3$^{-/-}$ and RIP3$^{-/-}$→WT mice were also similar to those of RIP3$^{-/-}$ mice (Fig. 2*C*). These data indicate that a lack of RIP3 in bone marrow cells, presumably in platelets, is responsible for the impaired thrombus formation in the RIP3$^{-/-}$ mice.

In the RIP3$^{-/-}$ mice, platelet size and morphology were normal (Table S1 and Fig. 2*D*), but the number of circulating platelets was slightly elevated (Fig. 2*E*). The numbers of α-granules and dense granules were comparable in the two genotypes (Fig. 2*F*). There were no significant differences in the surface expression of the major platelet glycoproteins (GP), CD42b (GPIbα), and CD41 (α_{IIb} subunit) between WT and RIP3$^{-/-}$ platelets (Fig. 2*G*).

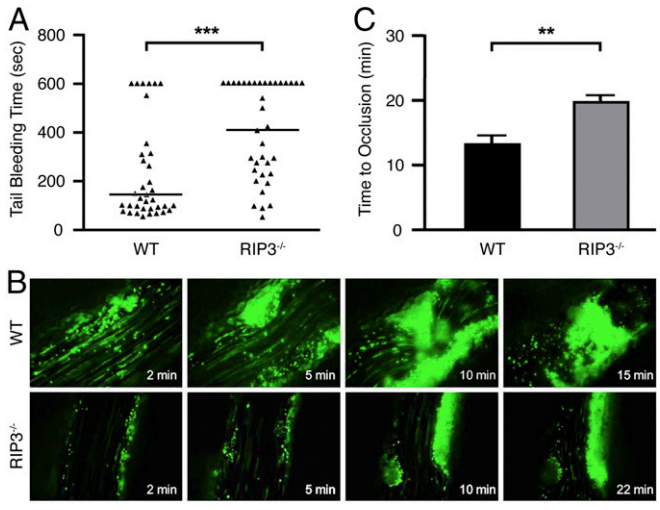

Fig. 1. The effects of RIP3 knockout on in vivo hemostasis and thrombosis. (*A*) Tail bleeding time determined in groups of 36 WT and 36 RIP3$^{-/-}$ mice. Horizontal lines indicated the median values. Statistical analysis was performed using the two-tailed Mann–Whitney test and revealed a significant difference between the two groups of animals. ***$P < 0.001$. (*B*) Representative images of $FeCl_3$-induced mesenteric arteriole thrombosis in WT and RIP3$^{-/-}$ mice as recorded by real-time microscopy. Time after $FeCl_3$-induced injury is indicated at the bottom right of each image. (*C*) Occlusion time of the mesenteric arteriole as measured by $FeCl_3$ injury in WT and RIP3$^{-/-}$ mice. Data are mean ± SEM values of 11 WT mice and 10 RIP3$^{-/-}$ mice. **$P = 0.0012$.

Impaired Platelet Aggregation Is Induced by U46619 and Thrombin in the Absence of RIP3. We next investigated the role of RIP3 in platelet function by examining the effect of RIP3 deficiency on agonist-induced platelet aggregation. Platelets lacking RIP3 showed a marked defect in aggregation in response to low concentrations of thrombin or a thromboxane A_2 analog, U46619 (Fig. 3 *A* and *B*). However, aggregation induced by collagen (Fig. 3*C*) and ADP (Fig. 3*D*) was not obviously different between WT and RIP3$^{-/-}$ platelets. To exclude the possibility that the decreased platelet aggregation induced by thrombin or U46619 is due to decreased expression levels of protease-activated receptor (PAR) 3/4 and TXA_2 (TP) receptors in RIP3$^{-/-}$ platelets, we assayed the expression levels of these receptors. We found that deletion of RIP3 did not affect the expression levels of PAR3/4 and TP in platelets (Fig. S1). These data indicate that RIP3 promotes U46619- and thrombin-induced platelet aggregation.

Impaired U46619 and Thrombin-Induced ADP Secretion Occur in the Absence of RIP3. ADP released from dense granules is known to play an important role in sensitizing platelets to low doses of agonist and amplifying platelet aggregation (21–24). Given our finding that RIP3 deficiency impaired platelet aggregation solely when low doses of the agonists were applied, we hypothesized that RIP3 might play a role in agonist-induced secretion. To test this hypothesis, we examined agonist-induced ATP release from RIP3$^{-/-}$ platelets. As shown in Fig. 4*A*, the second wave of ATP secretion elicited by low concentrations of U46619 was abolished in RIP3$^{-/-}$ platelets. Similarly, low-dose thrombin-induced ATP secretion was reduced in RIP3$^{-/-}$ platelets (Fig. 4*B*). In contrast, collagen-induced dense granule secretion was not impaired in RIP3$^{-/-}$ platelets (Fig. S2). These results indicate that RIP3 deficiency impairs dense granule secretion induced by U46619 or thrombin.

As shown in Fig. 3*D*, ADP-induced platelet aggregation was not impaired in RIP3$^{-/-}$ platelets, suggesting that RIP3 is not required for ADP-induced signaling. Therefore, we considered the possibility that the inhibitory effect of RIP3 deficiency on U46619- or thrombin-induced platelet aggregation results from inhibition of ADP secretion. In support of this idea, we found that the aggregation of WT platelets induced by U46619 or thrombin was reduced to a level comparable to that seen in RIP3-deficient platelets in the presence of apyrase, which hydrolyzes released ADP (Fig. S3). Moreover, the addition of a low concentration of ADP, although insufficient by itself to induce platelet aggregation, restored the reduced aggregation of RIP3$^{-/-}$ platelets stimulated with a low dose of U46619 or thrombin (Fig. 4 *C* and *D*). Therefore, these results indicate that RIP3 deficiency impairs U46619- and thrombin-induced ADP secretion.

α-Granule Secretion, TXA_2 Generation, Phosphatidylserine Exposure, and Integrin Activation Occur in RIP3$^{-/-}$ Platelets. Granule secretion and TXA_2 generation induced by various agonists play key roles in amplifying stimulation signals and enabling robust platelet

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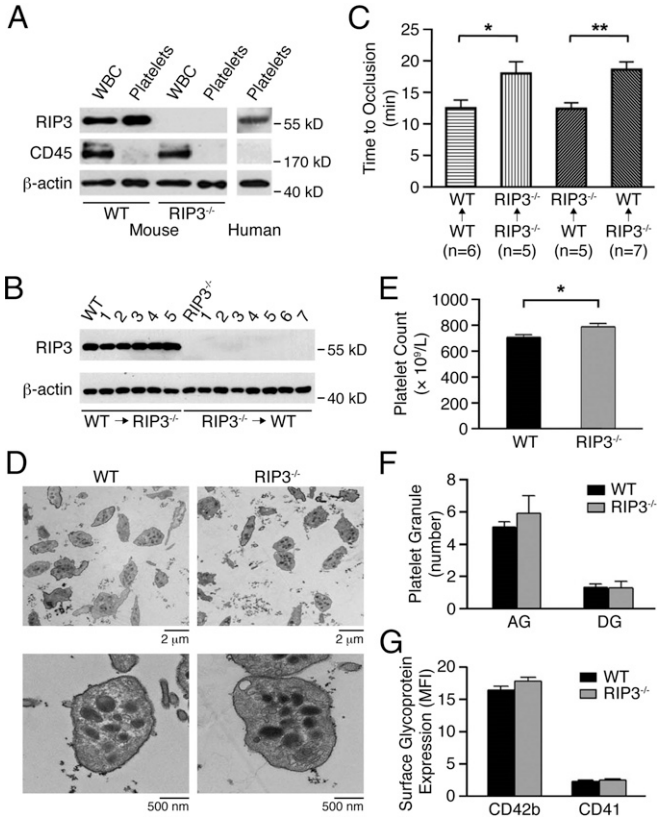

Fig. 2. Characterization of platelets from RIP3-deficient mice. (*A*) Whole blood and platelets were prepared as described in *SI Materials and Methods*. The washed platelets and leukocytes were lysed and probed by immunoblotting with antibodies against RIP3 and CD45 (marker for leukocytes). Blotting to β-actin was used as a lane loading control. The figures are representative of three independent experiments. (*B*) Expression of RIP3 in platelets from RIP3$^{-/-}$ (WT→RIP3$^{-/-}$; $n = 5$) and WT (RIP3$^{-/-}$ →WT; $n = 7$) mice repopulated with WT and RIP3$^{-/-}$ donor bone marrow- derived cells, respectively, was detected by Western blot analysis with anti-RIP3 antibody. Platelets from WT and RIP3$^{-/-}$ mice were used as controls, and β-actin served as a lane loading control. (*C*) Occlusion time of the mesenteric arteriole was measured by $FeCl_3$ injury in WT and RIP3$^{-/-}$ mice repopulated with either WT or RIP3$^{-/-}$ donor bone marrow- derived cells. Data are mean ± SEM values. $n = 5$~7. *$P = 0.035$; **$P = 0.0047$. (*D*) Electron microscopy analysis of WT and RIP3$^{-/-}$ mouse platelets. Representative images were obtained at 1,000× and 10,000×, respectively. (*E*) Platelet counts were detected in whole blood from WT and RIP3$^{-/-}$ mice ($n = 26$, equivalent numbers of males and females). Results are expressed as mean ± SEM. *$P < 0.05$. (*F*) Quantification of α-granules (AG) and dense granules (DG) was calculated from 20 platelets of five different fields of view for each genotype. Data are expressed as mean ± SEM. (*G*) Surface expression of the major glycoproteins on WT and RIP3$^{-/-}$ mouse platelets on membranes determined by flow cytometry with specific antibodies. Data are expressed as the mean fluorescence intensity (MFI) ± SEM of three different experiments.

activation at the site of injury. Thus, we further evaluated α-granule secretion in RIP3$^{-/-}$ platelets. We found that, consistent with a previous report (25), only very small amount of P-selectin exposure was detected in U46619-stimulated platelets (Fig. S4*A*). There was no significant difference in P-selectin exposure between WT and RIP3$^{-/-}$ platelets stimulated with thrombin (Fig. S4*B*). Moreover, no significant difference in PF4 release was observed between WT and RIP3$^{-/-}$ platelets stimulated with U46619 or thrombin (Fig. S5). These data suggest that RIP3 deficiency does not impair α-granule secretion.

We next investigated whether RIP3 was involved in TXA_2 synthesis by measuring the stable TXA_2 metabolite, TXB_2. We found no statistically significant difference in TXB_2 generation between WT and RIP3$^{-/-}$ platelets stimulated with thrombin or collagen (Fig. S6). Interestingly, ADP-induced TXB_2 production was markedly elevated in RIP3$^{-/-}$ platelets compared with in WT platelets (10.55 ± 1.97 ng/mL vs. 0.12 ± 0.05 ng/mL).

At the site of vascular injury, phosphatidylserine (PS) exposure on activated platelets provides a procoagulant surface to promote thrombus formation. There is a growing body of evidence suggesting that the biochemical, morphological, and functional changes underlying agonist-induced platelet procoagulant function are broadly consistent with cell necrosis (26). Therefore, we further investigated whether RIP3 deficiency impairs PS exposure in agonist-induced platelets. The results showed no obvious difference in PS exposure between WT and RIP3$^{-/-}$ platelets stimulated with different concentrations of thrombin or U46619 (Fig. S7).

In vitro platelet aggregation requires integrin $\alpha_{IIb}\beta_3$ activation. Thus, we investigated the role of RIP3 in integrin $\alpha_{IIb}\beta_3$ activation. We found that U46619-induced integrin $\alpha_{IIb}\beta_3$ activation was decreased in RIP3$^{-/-}$ platelets (Fig. S8*A*). Moreover, U46619-induced fibrinogen binding to platelets was reduced by the inhibition of PI3K-Akt signaling (2). Similarly, thrombin-induced integrin $\alpha_{IIb}\beta_3$ activation was decreased in RIP3$^{-/-}$ platelets as well (Fig. S8*B*). Furthermore, fibrinogen binding was reduced in the RIP3$^{-/-}$ platelets stimulated with thrombin (Fig. S8*C*).

RIP3 Regulates Akt Phosphorylation. It has been reported that PI3K-Akt signaling plays an essential role in regulating the second wave of ADP secretion in platelets in response to U46619 (2). Because RIP3 deficiency impairs only the second wave of ADP secretion, we investigated whether RIP3 regulates dense granule secretion via PI3K-Akt signaling. Phosphorylation of Akt

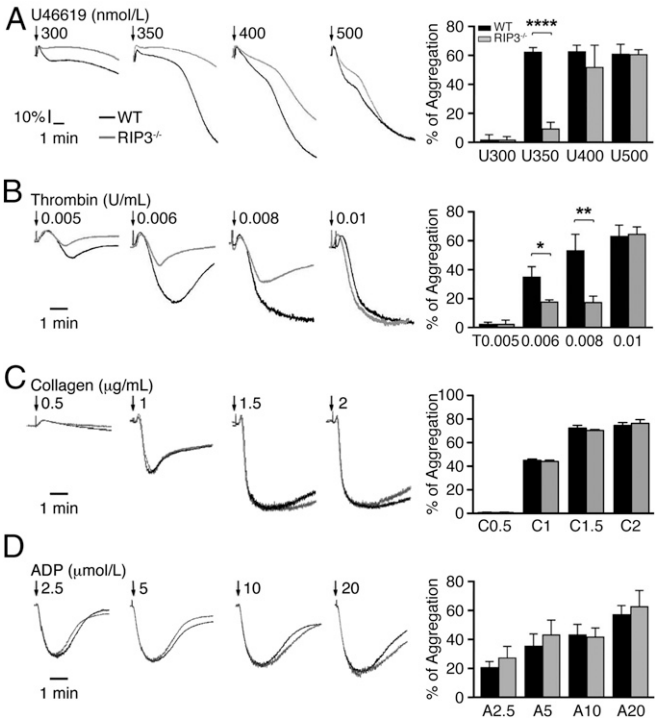

Fig. 3. The role of RIP3 in platelet aggregation. Washed platelets from WT and RIP3$^{-/-}$ mice were stimulated with different concentrations of U46619 (*A*), thrombin (*B*), collagen (*C*), and ADP (*D*) at 37 °C under constant stirring. Platelet aggregation was monitored using a turbidimetric aggregometer. The traces are representative of three independent experiments. In the histograms of maximal platelet aggregation under the indicated conditions, values are mean ± SEM of three independent experiments. *$P = 0.016$; **$P = 0.0071$; ****$P < 0.0001$.

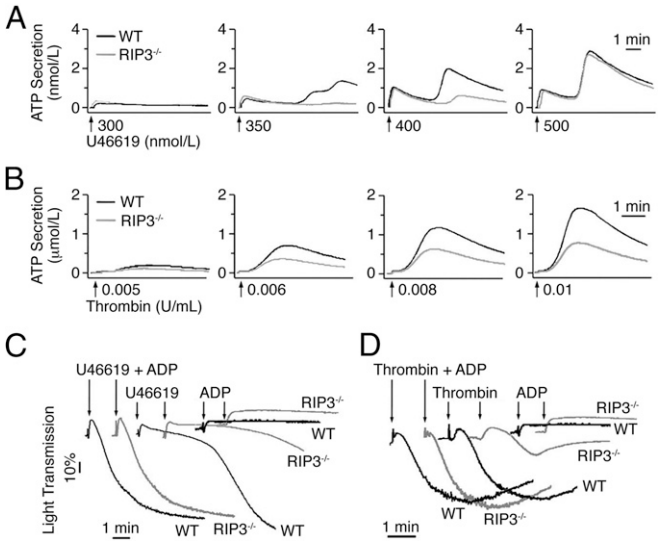

Fig. 4. ATP secretion of RIP3$^{-/-}$ platelets in response to low doses of U46619 or thrombin. (*A* and *B*) Washed platelets from WT and RIP3$^{-/-}$ mice were stimulated with different concentrations of U46619 (*A*) or thrombin (*B*). ATP secretion was recorded concomitantly with platelet aggregation in the presence of luciferin/luciferase reagent (80 nM). (*C* and *D*) A low concentration of ADP (0.25 μM), insufficient to induce aggregation, reversed the inhibitory effect of RIP3 deficiency on 325 nM U46619-induced (*C*) or 0.008 U/mL thrombin-induced (*D*) platelet aggregation. Trace in the figures are representative of at least three independent experiments.

at Thr^{308}, a known marker of Akt activation that lies downstream of PI3K (2, 27), was diminished from the onset of second wave of secretion in RIP3$^{-/-}$ platelets stimulated with U46619 (Fig. 5*A*). Moreover, low-dose thrombin-induced phosphorylation of Akt also was reduced from the onset of the second wave of secretion in RIP3$^{-/-}$ platelets (Fig. 5*B*). These data suggest that RIP3 is upstream of Akt regulating ADP secretion in platelets.

RIP3 Interacts with Gα$_{13}$ in Platelets. Both Gq and Gα$_{13}$ are required for platelet secretion induced by thrombin and TXA$_2$ (4, 28, 29). Because platelet activation elicited by ADP, which activates its receptors P2Y$_1$ and P2Y$_{12}$, which couple to Gq and Gi, respectively, was not reduced in the RIP3-deficient platelets, it is less likely that RIP3 is required for Gq signaling. Therefore, we hypothesized that RIP3 might be downstream of Gα$_{13}$ to regulate dense granule secretion. In support of this idea, we found that RIP3 could be coimmunoprecipitated with Gα$_{13}$ (Fig. 5*C*), but not with Gq or Gi (Fig. S9), from the lysates of platelets. Moreover, it appears that the interaction between RIP3 and Gα$_{13}$ is dynamically regulated on activation (Fig. S10).

To further characterize the specificity of the interaction between RIP3 and Gα$_{13}$, we first overexpressed RIP3 and Gα$_{13}$ proteins in HEK293T cells. We found that RIP3 was pulled down with Gα$_{13}$ from the HEK293T cells (Fig. 5*D*). We then examined the interaction between purified RIP3 and Gα$_{13}$ proteins by ELISA. Fig. 5*E* shows a specific interaction between RIP3 and Gα$_{13}$. To identify the binding site in RIP3 for Gα$_{13}$, we purified two fragments of RIP3, the N terminal (1–350 aa) and C terminal (351–518 aa). We found that, similar to the full-length RIP3, the C-terminal fragment interacted with Gα$_{13}$ protein, suggesting that the binding site in RIP3 for Gα$_{13}$ is located at the C terminal of RIP3 (Fig. 5*E*). To exclude the possibility of contamination with cellular proteins during purification, we further confirmed the interaction of RIP3 with Gα$_{13}$ by other sources of purified RIP3 and Gα$_{13}$ proteins (Fig. S11). In addition, we investigated whether the deletion of RIP3 affects the formation of G protein heterotrimer. We found no difference in the interaction of Gα$_{13}$ with Gβ between RIP3-deficient and WT platelets, suggesting that the formation of Gα$_{13}$ heterotrimer is not affected by RIP3 deletion (Fig. S12). Taken together, these data suggest that RIP3 regulates platelet activation by interacting with Gα$_{13}$.

RIP3 Plays a Role in Integrin Outside-In Signaling. It is known that integrin outside-in signaling is required to induce the second wave of secretion in platelets stimulated with U46619 (2). As shown in Fig. 5 *A* and *B*, Akt phosphorylation occurred at 3 min after stimulation, corresponding to initiation of the integrin outside-in signaling. Moreover, RIP3 was found to interact with Gα$_{13}$, which has been confirmed to play a key role in integrin outside-in signaling (5–7). Therefore, these findings suggest the involvement of RIP3 in integrin outside-in signaling. To test this possibility, we examined the effects of RIP3 deficiency on integrin outside-in signaling-dependent platelet spreading and clot retraction. We found markedly reduced spreading of RIP3$^{-/-}$ platelets on fibrinogen in the presence of thrombin compared with that of WT platelets (Fig. 6*A*). Furthermore, RIP3 deficiency impaired clot retraction, which requires integrin outside-in signaling (Fig. 6*B*). These findings suggest that RIP3 deficiency impairs integrin outside-in signaling.

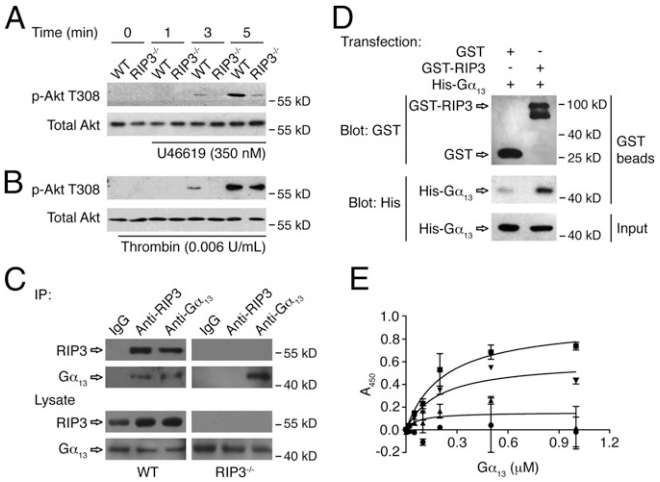

Fig. 5. The role of RIP3 in Akt phosphorylation, and the interaction of RIP3 with Gα$_{13}$. (*A* and *B*) Washed platelets were stimulated with 350 nM U46619 (*A*) or 0.006 U/mL thrombin (*B*) for indicated times at 37 °C, with stirring (1,000 rpm). Stimulated platelets were lysed and samples analyzed by Western blot with anti–phospho-Akt (Thr^{308}) and anti-total Akt antibodies. (*C*) Washed mouse platelets were lysed and immunoprecipitated with anti-mouse RIP3 and Gα$_{13}$ antibodies or IgG controls. After incubation with protein A/G plus agarose beads, the proteins were analyzed by Western blot with anti-RIP3 and anti-Gα$_{13}$ antibodies. (*D*) The pcDNA3.1(+)- expressing GST or GST-RIP3 and the pcDNA3.1(+)-expressing His-Gα$_{13}$ were cotransfected into HEK293T cells. The cells were cultured, harvested, and lysed. The lysates were centrifuged, after which the supernatants were mixed with glutathione beads and incubated at 4 °C overnight. The beads were washed, and the bead-bound proteins were analyzed by immunoblotting. The Western blot shown is representative of three independent experiments. (*E*) Purified proteins and BSA (4 μg/mL) were immobilized onto the wells of microtiter plates. Increasing concentrations of His-tagged human Gα$_{13}$ protein were incubated with immobilized GST (●), GST-RIP3 (■), GST-RIP3-N (▲), GST-RIP3-C (▼), and BSA. The binding of Gα$_{13}$ was detected by mouse anti-His antibody and HRP-conjugated goat anti-mouse antibody. The absorbance at 450 nm was measured in three independent experiments. Data are presented as mean ± SD after subtracting the binding of Gα$_{13}$ to BSA (negative control). The binding curve was fitted to the following equation: $Y = B_{max} \times x/(K_d + x)$, where Y is the specific binding, x is the ligand concentration, B_{max} is the binding maximum, and K_d is the equilibrium dissociation constant. For some data points, the error bars are smaller than the symbols.

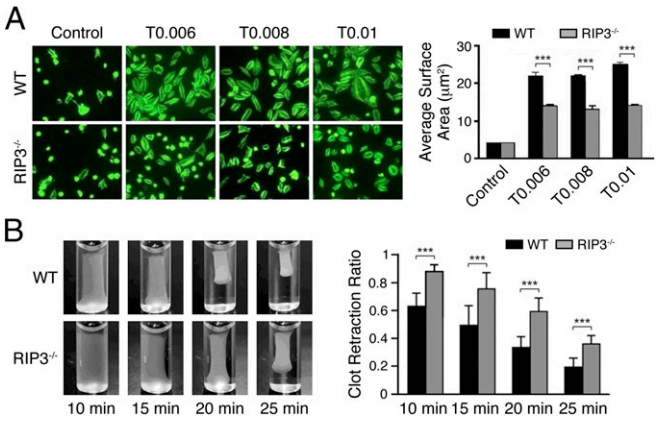

Fig. 6. RIP3 deficiency impairs integrin signaling. (*A*) Washed platelets were allowed to adhere and spread on fibrinogen-coated wells by thrombin or vehicle stimulation at 37 °C for 2 h. After being fixed, permeabilized, and stained, the platelets were observed with a fluorescence microscope. Images were acquired, and the spreading area of single platelets was measured using ImageJ2x software, with pixel number as the unit of size. (*Left*) Representative pictures. (*Right*) Surface areas of single platelets from 10 randomly selected fields of three different tests. ***$P < 0.001$. (*B*) Washed mouse platelets in modified Tyrode's buffer were mixed with 150 μg/mL purified human fibrinogen. The clots were initiated by the addition of human α-thrombin (1 U/mL) and incubation at 37 °C. (*Left*) Representative pictures of clot retraction from three separate experiments. (*Right*) 2D retraction of clots was measured, and the data are expressed as retraction ratio: 1 − (final clot size/initial clot size). $n = 3$. Data are mean ± SEM. ***$P < 0.001$, Student's t test.

To further examine whether the integrin outside-in signaling is defective in the absence of RIP3, we assessed the phosphorylation of ERK, which is the integrin signaling-dependent activation, in RIP3$^{-/-}$ platelets (30, 31). We found that ERK phosphorylation was obviously reduced in RIP3$^{-/-}$ platelets stimulated with U46619 or thrombin (Fig. S13). Moreover, consistent with Fig. 5*A*, we found that the integrin antagonist RGDS peptides abolished U46619-induced Akt phosphorylation in both WT and RIP3$^{-/-}$ platelets (Fig. S14). These data suggest that RIP3 regulates platelet activation via the regulation of integrin outside-in signaling.

RIP3 Inhibitor Blocks U46619- and Thrombin-Induced Human Platelet Aggregation and Prevents Arterial Thrombus Formation. To investigate the role of RIP3 in human platelets, we incubated an RIP3-specific inhibitor, GSK′872 (32), with human platelets. We found that GSK′872 dose-dependently inhibited U46619- and thrombin-induced human platelet aggregation (Fig. 7 *A* and *B*), but had no effect on the aggregation of RIP3$^{-/-}$ platelets (Fig. S15). To further characterize the effect of RIP3 inhibitor on in vivo thrombosis, we applied the $FeCl_3$-injured mesenteric arteriole thrombosis model using mice injected with GSK′872. Compared with vehicle-injected controls, the mice injected with GSK′872 exhibited delayed and diminished thrombus formation (Fig. 7*C*). Occlusion times were significantly prolonged in the mice injected with GSK′872 (29.2 ± 2.5 min in the mice injected with GSK′872 vs. 18.5 ± 0.9 min in the vehicle control mice; $P < 0.01$) (Fig. 7*D*). These data demonstrate the role of RIP3 in human platelets, and suggest the antithrombotic potential of RIP3 inhibitor.

Discussion

In this study, we have demonstrated that RIP3 is expressed in platelets and plays important roles in arterial thrombus formation and hemostasis in vivo. We report that platelet aggregation and dense granule secretion in response to U46619 or thrombin, spreading on fibrinogen, and clot retraction are impaired in RIP3-deficient platelets, and that RIP3 antagonist inhibits platelet aggregation and prevents in vivo arterial thrombus formation.

Our data show that RIP3 deficiency selectively impairs U46619- and thrombin-induced dense granule secretion and aggregation. ADP-induced aggregation is not affected by RIP3 deletion. Because both the TXA_2 receptor and the thrombin receptor couple to $G\alpha_{13}$, we hypothesize that RIP3 may be downstream of $G\alpha_{13}$ and mediate $G\alpha_{13}$ signaling. In support of this hypothesis, we found that RIP3 interacts with $G\alpha_{13}$ in platelets. Moreover, consistent with our findings, deletion of $G\alpha_{13}$ selectively impairs low doses of thrombin- and U46619-induced platelet aggregation (4), but does not affect ADP-induced aggregation (33).

Four lines of evidence from the present study indicate that RIP3 regulates integrin outside-in signaling. First, the integrin signaling-dependent second wave of dense granule secretion and aggregation was diminished in RIP3-deficient platelets. Second, RIP3 deficiency impaired platelet spreading on fibrinogen and clot retraction. Third, ERK phosphorylation, downstream of integrin signaling (30, 31), was reduced in RIP3-deficient platelets. Fourth, RGDS, the integrin-specific antagonist, abolished the second wave of dense granule secretion and Akt phosphorylation in both WT and RIP3$^{-/-}$ platelets. These findings are consistent

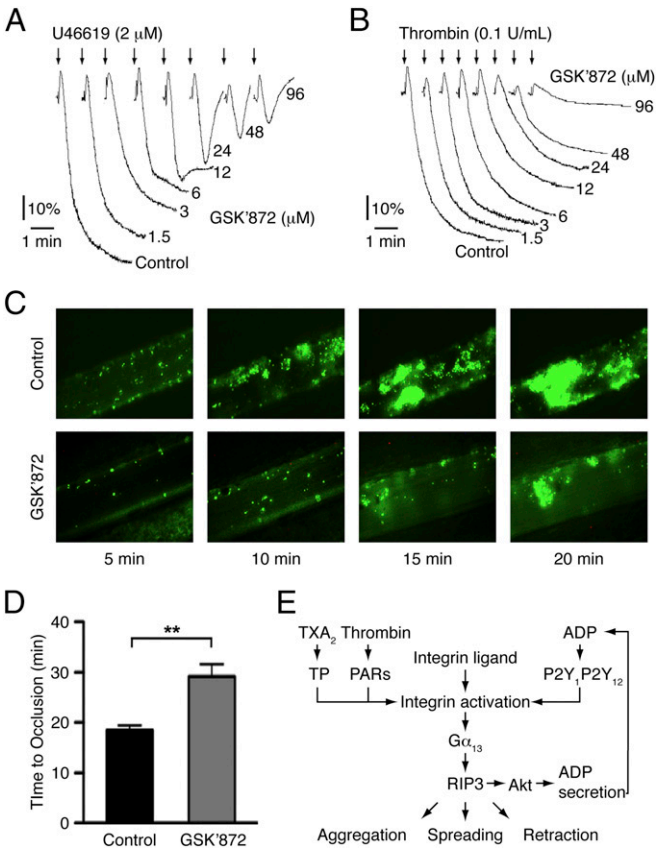

Fig. 7. RIP3 inhibitor GSK′872 blocks human platelet aggregation and in vivo thrombus formation. (*A* and *B*) Washed human platelets were incubated with different concentrations of GSK′872 or vehicle (DMSO) at 37 °C for 30 min, and then stimulated with U46619 (*A*) and thrombin (*B*). Platelet aggregation was monitored using a turbidimetric aggregometer. The traces are representative of 3 independent experiments. (*C*) Representative images of $FeCl_3$-induced mesenteric arteriole thrombosis in C57d mice injected with GSK′872 or vehicle (DMSO) as recorded by real-time microscopy. Time after $FeCl_3$-induced injury is indicated at the bottom right of each image. (*D*) The occlusion time of mesenteric arteriole injured by $FeCl_3$ in C57d mice injected with GSK′872 or vehicle. Data are mean ± SEM values of 15 controls and 11 GSK′872-injected mice. **$P < 0.01$. (*E*) RIP3, interacting with $G\alpha_{13}$, regulates integrin outside-in signaling.

with the recent reports demonstrating that $G\alpha_{13}$ binds to integrin $\alpha_{IIb}\beta_3$ and mediates integrin outside-in signaling (5–7). RIP3 was found to interact with $G\alpha_{13}$, thereby providing a molecular basis for the involvement of RIP3 in $G\alpha_{13}$-mediated integrin signaling (Fig. 7*E*).

We found that integrin activation was reduced in the RIP3-knockout platelets. Akt phosphorylation elicited by thrombin or U46619 was decreased in the absence of RIP3. These findings are consistent with previous reports indicating that deletion of Akt1 (22) or Akt2 (21), or inhibition of Akt (2), reduced thrombin- or U46619-induced fibrinogen binding. Because our data indicate impairment of dense granule secretion in RIP3 knockout mice, future work is needed to determine whether the impaired integrin activation in RIP3-deficient platelets is due to a direct role of RIP3 on integrin or results from reduced platelet secretion.

Activation of RIP3 phosphorylates its substrate, such as MLKL, leading to necrotic death and clearance of the target cells (13–15). In contrast, we demonstrate here that RIP3 promotes platelet activation and thrombus formation independent of MLKL. These findings identify RIP3 as an important regulator of platelet activation and thrombosis. In particular, the RIP3 inhibitor effectively prevented arterial thrombus formation in vivo, suggesting a novel potential antithrombotic strategy for thrombotic diseases.

In summary, our data demonstrate a critical role for RIP3 in promoting hemostasis and thrombus formation in vivo. RIP3 amplifies U46619- and thrombin-induced platelet activation by regulating integrin outside-in signaling. RIP3 antagonist inhibits human platelet aggregation in vitro and prevents arterial thrombus formation in vivo. Therefore, RIP3 may represent a signaling molecule to modulate platelet activation and a promising therapeutic target for thrombotic diseases.

Materials and Methods

Reagents, electron microscopy, measurement of TXA_2 generation, flow cytometry analysis, hematologic analysis, tail bleeding time (34), in vivo thrombosis (35), irradiation and bone marrow-derived cell repopulation, platelet aggregation and secretion, assessment of fibrinogen binding, coimmunoprecipitation, plasmids and oligos, expression and purification of proteins, ELISA, transfection and protein-binding assays, platelet spreading on immobilized fibrinogen, and clot retraction are described in *SI Materials and Methods*.

Mice. RIP3-deficient ($RIP3^{-/-}$) mice were generated in the laboratory of Dr. Xiaodong Wang (11), and the 129/B6 $RIP3^{-/-}$ mice have been backcrossed to C57/B6 for 10 generations. WT control mice and RIP3-deficient mice used in this study were littermates generated from heterozygous breeding. All of the animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

Platelet Preparation. Platelets from healthy volunteers were prepared as described previously (2). For studies involving human subjects, approval was obtained from the Ethics Committee of the First Affiliated Hospital of Soochow University. Written informed consent was provided by each particpant, and the studies were performed in accordance with the Declaration of Helsinki. Washed platelets from mice were prepared as described previously (2).

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