A Phosphoinositide 3-Kinase-AKT-Nitric Oxide-cGMP Signaling Pathway in Stimulating Platelet Secretion and Aggregation*

Phosphoinositide 3-kinase (PI3K) and Akt play important roles in platelet activation. However, the downstream mechanisms mediating their functions are unclear. We have recently shown that nitric-oxide (NO) synthase 3 and cGMP-dependent protein kinase stimulate platelet secretion and aggregation. Here we show that PI3K-mediated Akt activation plays an important role in agonist-stimulated platelet NO synthesis and cGMP elevation. Agonist-induced elevation of NO and cGMP was inhibited by Akt inhibitors and reduced in Akt-1 knock-out platelets. Akt-1 knock-out or Akt inhibitor-treated platelets showed reduced platelet secretion and aggregation in response to low concentrations of agonists, which can be reversed by low concentrations of 8-bromo-cGMP or sodium nitroprusside (an NO donor). Similarly, PI3K inhibitors diminished elevation of cGMP and inhibited platelet secretion and the second wave platelet aggregation, which was also partially reversed by 8-bromo-cGMP. These results indicate that the NO-cGMP pathway is an important downstream mechanism mediating PI3K and Akt signals leading to platelet secretion and aggregation. Conversely, the PI3K-Akt pathway is the major upstream mechanism responsible for activating the NO-cGMP pathway in platelets. Thus, this study delineates a novel platelet activation pathway involving sequential activation of PI3K, Akt, nitric-oxide synthase 3, sGC, and cGMP-dependent protein kinase.

Platelets play a critical role in thrombosis and hemostasis. At sites of vascular injury, platelets are activated by various soluble agonists such as thrombin and ADP and adhesive proteins such as collagen and von Willebrand factor. Although different agonists induce platelet activation via different signaling pathways, the signals induced by different agonists converge to common signaling events such as calcium mobilization and activation of the ligand binding function of the integrin $\alpha_{IIb}\beta_3$ that mediates platelet aggregation (1, 2). An important feature of platelet activation is the ability to self-amplify the signals, which allows low concentrations of agonists to induce maximal platelet responses. This feature is particularly important in arteries where fast flow of blood may quickly dilute soluble agonists. One important mechanism of self-amplification is the secretion of platelet granule contents such as platelet agonists ADP and serotonin and adhesive proteins von Willebrand factor and fibrinogen (3). The secreted platelet agonists and adhesive proteins, via various pathways, form “positive feedback loops” that greatly amplify and stabilize platelet aggregation, thus sensitizing platelets to low doses of platelet agonists. The signaling mechanism leading to platelet granule secretion is not totally understood. We have recently shown that nitric oxide (NO)3 synthesized by NO synthase 3 (NOS3, also called eNOS) stimulates soluble guanylyl cyclase and induces cGMP elevation and activation of cGMP-dependent protein kinase (PKG), leading to secretion of platelet granules and the second wave of platelet aggregation (4–6). Furthermore, our findings show that the NO-cGMP-PKG pathway plays a time- and concentration-dependent biphasic role in platelet activation, a stimulatory role at low concentrations of NO and cGMP synthesized following agonist stimulation, and an inhibitory role when platelets are exposed to high concentrations of NO and cGMP (4, 6, 7).

There has been evidence for several years that phosphoinositide 3-kinase (PI3K) is involved in platelet activation, particularly at low concentrations of agonists (8–12). We have recently shown that PI3K plays an important role in signaling the aggregation-dependent secretion and thus the secretion-dependent second wave of platelet aggregation (11). PI3K, via phosphatidylinositol-dependent kinase-1 activates Akt, which is a family of intracellular serine/threonine protein kinases (also called protein kinase B) (for reviews, please see Refs. 13–15). There are three isoforms of Akt: Akt-1, Akt-2, and Akt-3. Recent studies show that both Akt-1 and Akt-2 are important in low dose agonist-induced platelet activation and in platelet-dependent thrombus formation in vivo (16, 17). However, the downstream signaling pathways that are responsible for PI3K- and Akt-mediated platelet activation have been unclear. Although Akt-1 has been shown to phosphorylate and activate NOS3 in endothelial cells (18), the possibility that Akt may promote platelet secretion and aggregation via the NOS-sGC pathway has not been considered, partly because the dogma for the past 30 years has been that NO inhibits platelet activation. In this study, we present data showing that the PI3K-Akt pathway stimulates platelet NO synthesis and cGMP elevation during platelet activation and stimulates platelet secretion and secretion-dependent second wave platelet aggregation by activating the NO-cGMP pathway. Together with our recent findings, this study delineates a novel platelet activation signaling pathway in which platelet agonists activate PI3K and thus Akt, which stimulates NO synthesis and cGMP elevation, activates PKG, and induces secretion of granule contents such as ADP, thus amplifying and stabilizing platelet aggregation.
An Akt-NO-cGMP Pathway Stimulates Platelet Activation

EXPERIMENTAL PROCEDURES

Materials and Animals—Thromboxane A2 analog U46619, membrane-permeable cGMP analog 8-bromo-cGMP, PI3K inhibitors wortmannin and Ly294002, and Akt inhibitor SH-6 (19) and Akt inhibitor 1 were purchased from Calbiochem. In some experiments, 8-bromo-cGMP from Axoora (San Diego, CA) was also used. Human α-thrombin was from Enzyme Research Laboratories, South Bend, IN. Sodium nitroprusside (SNP) was obtained from Sigma. Collagen was from Chronolog, Havertown, PA. The generation of Akt-1 knock-out mice has been previously described (20). Akt-1 knock-out mice are on a mixed 129R1(50%)/C57BL (50%) background. Wild type control mice and Akt-1-/- mice used in this study were 8–15-week-old littermates generated from heterozygous breeding (20).

Platelet Aggregation and Secretion—Preparation of washed human platelets has been previously described (11). For preparation of mouse platelets, blood from 5–6 mice of either genotype was pooled and platelets were isolated and washed as previously described (11). Final concentration of resuspended platelets was 3 × 10^7/ml. Platelets were allowed to rest for at least 1 h at 22 °C before use. Platelet aggregation and secretion of granule ATP were determined simultaneously in a Chronolog lumiaggregometer at 37 °C with stirring (1000 rpm) after addition of the luciferin-luciferase reagent and platelet agonists (6). To investigate the upstream mechanism of NOS activation during platelet activation, we examined the synthesis and PKG activation (6). To investigate the upstream mechanism of NOS activation during platelet activation, we examined the synthesis and PKG activation (6).

RESULTS

The Role of Akt in the Activation of Platelet Nitric Oxide Synthesis—We have recently shown that NOS 3 plays a stimulatory role in platelet activation by producing nitric oxide, thus stimulating cGMP synthesis and PKG activation (6). To investigate the upstream mechanism of NOS activation during platelet activation, we examined the role of Akt-1 in agonist-stimulated NO synthesis in platelets. Platelet agonists such as thrombin and collagen induce elevation of platelet NO levels. Fig. 1 shows that NO production stimulated by thrombin was significantly reduced in Akt-1-/- platelets. Similarly, collagen-induced NO production was inhibited in Akt-1-/- platelets, suggesting that Akt-1 plays an important role in stimulating NO production during platelet activation in mice. To investigate whether Akt and PI3K play a role in human platelet NO production, human platelets were preincubated with Akt inhibitor SH6 (Fig. 1) and then stimulated with collagen. Platelet NO production was significantly inhibited by these inhibitors (Fig. 1C), indicating that Akt and
PI3K are important in the activation of human platelet NO synthesis. Interestingly, although NO production was dramatically reduced, NO was still detectable in either Akt-1−/− platelets or Akt inhibitor-treated platelets stimulated with agonists. These results suggest the possible presence of an Akt-1-independent NO synthesis mechanism but do not exclude the roles of other Akt isoforms or possible incomplete inhibition of PI3K or Akt by inhibitors. These data indicate that Akt-1 is an important, but not the only, signal mediator in the activation of platelet NO synthesis.

The Role of Akt and PI3K in Agonist-induced cGMP Production—A major role for NO in platelets is to stimulate soluble guanylyl cyclase that synthesizes cGMP. Therefore, we investigated whether Akt is important in agonist-induced cGMP elevation. Fig. 2 shows that the platelet agonists collagen, thrombin, and U46619 (a thromboxane A2 analog) induced significant elevation of cGMP in mouse and human platelets, which is consistent with results from previous studies (4, 5, 22, 23). Agonist-induced cGMP elevation was significantly reduced in Akt-1−/− mouse platelets (Fig. 2, A–C) and was also inhibited in human platelets treated with the Akt inhibitor SH6 (Fig. 2, D–F). These data indicate that Akt is indeed important in platelet cGMP elevation induced by platelet agonists. Because it is known that Akt-1 is activated by PI3K, we also examined the effect of a PI3K inhibitor, wortmannin, on agonist-induced cGMP elevation. Fig. 3 shows that cGMP elevation was inhibited by wortmannin. Thus, the PI3K-Akt pathway stimulates platelet cGMP elevation by activating platelet NO synthesis.

Reduced Platelet Responses in Akt-1−/− Mouse Platelets and Rescue by cGMP—To determine whether the role of Akt in stimulating platelet NO and cGMP elevation is a mechanism responsible for its stimulatory role in platelet activation, we investigated the responses of Akt-1 knock-out platelets to platelet agonists, collagen, thrombin, and U46619. Compared with wild type platelets, Akt-1 knock-out mouse platelets showed reduced platelet aggregation in response to low concentrations of collagen and thrombin and a significantly retarded aggregation response to U46619 (Fig. 4). At high concentrations of agonists, the inhibitory effect of Akt knock out on platelet aggregation was not significant. These results indicate a role for Akt-1 in low dose agonist-induced platelet aggregation, consistent with previous studies indicating the stimulatory role of PI3K and Akt in platelet activation (11, 16, 17). The reduction in platelet aggregation in Akt-1−/− platelets is characteristic of reduced secretion-dependent secondary platelet activation and is similar to the reduced platelet secretion and aggregation in platelets with deficiencies in NOS3 or PKG (5, 6). Indeed, platelet secretion of ATP was significantly reduced in Akt-1-knock-out platelets (Fig. 4). These results suggest that, similar to platelet defects caused by PKG- or NOS3-knock-out, the Akt-1-knock-out platelets are mainly defective in platelet secretion and secretion-dependent secondary platelet aggregation. To exclude the possibility that the defect in ATP secretion is caused by the reduced total ATP in Akt-1−/− platelets, we have also determined total ATP contents in wild type and Akt-1−/− platelets, which are consistent with previous studies indicating the stimulatory role of PI3K and Akt in platelet activation (11, 16, 17).

Role of PI3K in thrombin-induced cGMP production. Washed human platelets were treated with PI3K inhibitor wortmannin (100 nM, dissolved in Me2SO) or with Me2SO (0.1%) and then stimulated with thrombin (0.02 units/ml) at 37 °C in a platelet aggregometer for 5 min. After stopping the reaction, platelet cGMP levels were determined using an enzyme-linked immunosassay assay as described under “Experimental Procedures.” D–F, washed human platelets (5 × 109/ml) were preincubated with SH6 (20 μM, dissolved in Me2SO) or 0.067% Me2SO (control) for 2 min and then placed in the platelet aggregometer. After stimulation with indicated agonists (thrombin, 0.02 units/ml; U46619, 500 nM; collagen, 0.5 μg/ml), platelet cGMP was measured as described in panels A–C. Bars indicate mean ± S.D.

FIGURE 2. The role of Akt in mediating agonist-induced platelet cGMP elevation. A–C, washed platelets (3 × 109/ml) from Akt-1+/+ or Akt-1−/− mice were stimulated with indicated agonists (thrombin, 0.015 units/ml; U46619, 200 nM; collagen, 0.5 μg/ml) in a platelet aggregometer at 37 °C for 5 min. After stopping the reaction, platelet cGMP levels were determined using an enzyme-linked immunosassay assay as described under “Experimental Procedures.” D–F, washed human platelets (5 × 109/ml) were preincubated with SH6 (20 μM, dissolved in Me2SO) or 0.067% Me2SO (control) for 2 min and then placed in the platelet aggregometer. After stimulation with indicated agonists (thrombin, 0.02 units/ml; U46619, 500 nM; collagen, 0.5 μg/ml/ml), platelet cGMP was measured as described in panels A–C. Bars indicate mean ± S.D.
factors: the membrane-permeable cGMP analog may differ from endogenous cGMP in concentration, the site and timing of elevation, affinity for cGMP-binding proteins, or degradation by phosphodiesterases. The partial reversal may also indicate a possible cGMP-independent downstream pathway of Akt-1. Nevertheless, the significant reversal by the cGMP analog suggests that the NO and cGMP pathway is one of the important signaling mechanisms downstream of Akt-1 in promoting platelet secretion and aggregation.

Inhibition of Platelet Secretion and Aggregation by a Akt Inhibitor—To confirm the role of Akt in cGMP-dependent platelet activation, we tested the effects of the Akt inhibitors SH6 and Akt inhibitor 1 on platelet activation. SH6 (Fig. 5) and Akt inhibitor 1 (not shown) inhibited platelet aggregation and secretion induced by low concentrations of platelet agonists thrombin, U46619, and collagen in human and mouse (not shown) platelets. As with the Akt-1−/− platelets, 8-bromo-cGMP significantly reversed the inhibitory effects of SH6 (Fig. 5) and Akt inhibitor 1 (not shown). Similar to the effect of the cGMP analog on Akt-1−/− platelets, the ability of cGMP to reverse the effect of SH6 or Akt inhibitor 1 only occurred when low concentrations of cGMP analogs were used. The concentrations of cGMP analog required for rescue varied with different agonists and different donors in different experiments. High concentrations of 8-bromo-cGMP, as previously described, inhibited platelet aggregation (not shown, cf. Fig. 4B), which is consistent with our previous results that cGMP plays biphasic roles in platelet activation (4, 6, 7). Thus, the results obtained with either Akt-1 knock-out platelets or platelets treated with Akt inhibitors are consistent and show that Akt is an important component of the signaling mechanisms upstream of the cGMP signaling pathway. These results also suggest that human and mouse platelets share a similar dependence on an Akt-mediated pathway that is responsible for cGMP elevation and thus promotes cGMP-dependent platelet secretion and aggregation.

Akt Inhibitor-induced Platelet Inhibition Is Reversed by an NO Donor—To further verify that NO-mediated cGMP elevation is downstream of Akt in promoting platelet activation, we determined whether...
the NO donor SNP reversed the effect of Akt inhibitor SH6. Similar to 8-bromo-cGMP, low concentrations of SNP significantly but partially reversed the inhibitory effects of SH6 on platelet secretion and aggregation in response to three different platelet agonists (Fig. 6A). The difference in platelet aggregation with or without adding SNP was statistically significant (p < 0.05) or highly significant (p < 0.01) in all the experiments shown as analyzed using a paired t test. Typical platelet secretion and aggregation traces are shown. B, human platelets treated with MeSO (control) or SH6 were stimulated with thrombin, immediately followed by addition of increasing concentrations of SNP. Platelet ATP secretion and aggregation traces were recorded as above. Please note the concentration-dependent biphasic effects of SNP.

Effects of cGMP Analogs in Rescuing the PI3K Inhibitor-induced Platelet Inhibition—Akt is activated by PI3K, which is also known to play an important role in mediating platelet secretion and the secretion-dependent second wave of platelet aggregation (11). To investigate the importance of PI3K in cGMP signaling, human platelets were pretreated with wortmannin and then stimulated with thrombin. As reported previously (11), wortmannin diminished platelet secretion and the secretion-dependent second wave of platelet aggregation (Fig. 7). The wortmannin-induced reduction in platelet secretion and aggregation was partially rescued by low concentrations of 8-bromo-cGMP (Fig. 7), suggesting that cGMP is downstream of PI3K and contributes to the mechanism by which PI3K promotes platelet aggregation.

DISCUSSION

The data presented here define an important role for PI3K and Akt in stimulating platelet secretion and aggregation via the activation of the NO-cGMP signaling pathway. This study also links two apparently different platelet signaling pathways, the PI3K-Akt and the NO-cGMP pathways, and reveals the importance of this linkage in mediating platelet granule secretion and in amplifying and stabilizing platelet aggregation.

The finding that the NO-cGMP pathway serves as one of the impor-
An Akt-NO-cGMP Pathway Stimulates Platelet Activation

**FIGURE 7.** Reversal of wortmannin-induced inhibition of platelet secretion and aggregation by 8-bromo-cGMP and a schematic diagram of a novel Akt-dependent platelet activation pathway. A, washed platelets were pretreated with wortmannin (100 nM) or Me$_2$SO for 1 min. These platelets were then stimulated with thrombin (0.03 units/ml) with or without addition of 8-bromo-cGMP (1 μM; Calbiochem) immediately following thrombin. Real-time ATP secretion and platelet aggregation were simultaneously recorded. The experiments were performed at least three times using different donors. The difference in platelet aggregation with or without adding 8-bromo-cGMP was statistically significant (p < 0.05) as analyzed using a paired Student’s t test. Typical platelet secretion and aggregation traces are shown. B, a novel Akt-dependent signaling pathway in which platelet agonists sequentially activate PI3K, Akt, NOS3, sGC, and cGMP-dependent protein kinase, leading to platelet secretion and amplification and stabilization of platelet aggregates.

Tant downstream mediators of the PI3K-Akt pathway is a significant advance in our understanding of the roles of PI3K-Akt pathway in platelet activation. Several previous studies indicated that PI3K promotes platelet activation. PI3K inhibitors inhibited platelet aggregation (8, 11). Platelets from mice lacking different isoforms of PI3K showed reduced responses to low concentrations of platelet agonists (10–12). In many cell types, the protein kinase Akt has been identified as a major downstream effector of PI3K (13–15, 24, 25). PI3K also stimulates Akt activation during platelet activation (11). Consistent with the role of Akt in transmitting PI3K-mediated signals, two different isoforms of Akt, Akt1 and Akt2, have been found to be important in platelet activation (16, 17). The downstream mediators of the PI3K-Akt pathway have been unclear. Early studies suggested that PI3K promotes integrin activation in platelets (8, 9). However, the defects in platelet aggregation caused by inhibitors of either PI3K or Akt as well as the defects in platelets from PI3K or Akt knock-out mice showed a characteristic of inhibition in the secretion-dependent second wave of platelet aggregation (8, 11, 16, 17). Furthermore, we showed recently that PI3K in fact plays an important role in stimulating aggregation-dependent platelet secretion and thus amplifies the integrin-activation signal, inducing the second wave of platelet aggregation (11). Several signaling molecules have been implicated as the downstream targets of Akt (13–15). These include glycogen synthase kinase-3β, p70S6 kinase, mammalian target of rapamycin (mTOR), and NOS3 (18). Although the roles for most of these molecules in platelet activation are not clear, we have recently found that the NOS3-generated NO induces cGMP production by soluble guanylyl cyclase and activates the PKG-dependent platelet secretion pathway (6). Here we have shown that Akt induces NO synthesis and cGMP elevation during platelet activation and thus promotes platelet secretion and aggregation, as Akt-1$^{-/-}$ platelets or Akt inhibitor-treated platelets were defective in NO and cGMP elevation, platelet secretion, and the second-wave platelet aggregation induced by platelet agonists. Furthermore, the inhibitory effect of Akt knock-out or Akt inhibitors was corrected by supplementation with low concentrations of 8-bromo-cGMP or NO donor, SNP. Therefore, an important mechanism by which Akt stimulates platelet secretion is to activate NOS3 and the cGMP-dependent protein kinase pathway.

The conclusion that the NO-cGMP pathway is an important downstream mediator of the PI3K-Akt signals in platelet activation does not exclude the possibility that PI3K and/or Akt may have other downstream effector pathways. In fact, the defect in platelet secretion and aggregation in Akt-1$^{-/-}$ platelets or in SH6-treated platelets were only partially corrected by supplementation with cGMP analogs and NO donors. It is possible that NO donor SNP or the membrane-permeable cGMP analog may not completely substitute for the endogenously produced NO and cGMP due to the differences in structure, location, and timing of elevation, etc. However, the observation of partial restoration of platelet aggregation by NO donors and cGMP analogs in Akt-1$^{-/-}$ platelets may also suggest the possibility that other downstream effectors of Akt are important in Akt-mediated platelet activation.

Although the role of PI3K and Akt in stimulating platelet activation is generally accepted, the role of NO and cGMP in platelet activation has been controversial (26). Although early studies suggested a stimulatory role for cGMP (22, 23, 27), it has been dogma for the past 30 years that NO and cGMP play inhibitory roles in platelet activation, as it was demonstrated that high concentrations of NO donors and cGMP analogs inhibited platelet function (28–31). However, we have recently found that low concentrations of endogenously synthesized NO and cGMP in fact promote platelet secretion and aggregation (4, 5). These apparently controversial results are well explained by the difference in the concentrations and timing of the NO donors and cGMP analogs used in different studies (4, 7). Our results suggest that NO and cGMP play a biphasic role in platelet activation: a stimulatory role at low concentrations of cGMP induced during platelet activation and an inhibitory role at high concentrations. In addition, a major mechanism by which the NO-cGMP pathway stimulates platelet activation is to induce platelet secretion and the secretion-dependent second wave of platelet aggregation (5). Thus, the in vitro effect of NO and cGMP on platelet activation is apparent only when low dose platelet agonists are used, with which aggregation-dependent secretion is required for full-scale
platelet aggregation. In contrast, some previous studies used platelets that failed to respond to low dose platelet agonists and showed aggregation traces characteristic of a lack of platelet secretion (32, 33). Because high dose agonist-induced platelet aggregation as reported in such studies did not require aggregation-dependent platelet secretion, it is reasonable that the role of the NO-cGMP pathway in secretion would not be manifested under these conditions. Because the stimulatory roles of PI3K and Akt in platelet activation are known, the present finding that the PI3K-Akt pathway is the major upstream activator of the NOS and requires NO and cGMP for its stimulatory effect provides further support for the stimulatory role of agonist-induced endogenous NO and cGMP production in platelet activation. More importantly, our findings delineate a novel signaling pathway that stimulates platelet granule secretion and aggregation. In this new pathway, platelet agonists sequentially activate PI3K, Akt, and NOS3, which synthesizes NO. NO stimulates guanylyl cyclase, which elevates intracellular cGMP levels. Elevation of cGMP activates PKG, which activates mitogen-activated protein kinases (34, 35) and stimulates platelet granule secretion (5). Secreted granule contents such as ADP amplify platelet activation signals, leading to the further activation of platelet integrins and the second wave of platelet aggregation (Fig. 7B). Platelet secretion is also likely to contribute to the development of atherosclerosis.

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