PDK1 Determines Collagen-Dependent Platelet Ca$^{2+}$ Signaling and Is Critical to Development of Ischemic Stroke In Vivo

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Objective—Activation of platelets by subendothelial collagen results in an increase of cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]i) and is followed by platelet activation and thrombus formation that may lead to vascular occlusion. The present study determined the role of phosphoinositide-dependent protein kinase 1 (PDK1) in collagen-dependent platelet Ca$^{2+}$ signaling and ischemic stroke in vivo.

Approach and Results—Platelet activation with collagen receptor glycoprotein VI agonists collagen-related peptide or convulxin resulted in a significant increase in PDK1 activity independent of second-wave signaling. PDK1 deficiency was associated with reduced platelet phospholipase Cγ2–dependent inositol-1,4,5-trisphosphate production and intracellular [Ca$^{2+}$]i in response to stimulation with collagen-related peptide or convulxin. The defective increase of [Ca$^{2+}$]i resulted in a substantial defect in activation-dependent platelet secretion and aggregation on collagen-related peptide stimulation. Furthermore, Rac1 activation and spreading, adhesion to collagen, and thrombus formation under high arterial shear rates were significantly diminished in PDK1-deficient platelets. Mice with PDK1-deficient platelets were protected against arterial thrombotic occlusion after FeCl3-induced mesenteric arteries injury and ischemic stroke in vivo. These mice had significantly reduced brain infarct volumes, with a significantly increased survival of 7 days after transient middle cerebral artery occlusion without increase of intracerebral hemorrhage. Tail bleeding time was prolonged in pdk1−/− mice, reflecting an important role of PDK1 in primary hemostasis.

Conclusions—PDK1 is required for Ca$^{2+}$-dependent platelet activation on stimulation of collagen receptor glycoprotein VI, arterial thrombotic occlusion, and ischemic stroke in vivo. (Arterioscler Thromb Vasc Biol. 2016;36:1507-1516. DOI: 10.1161/ATVBAHA.115.307105.)

Key Words: Ca2+ signaling ■ ischemic stroke ■ PDK1 ■ platelets ■ PLCγ2

Platelet adhesion, activation, and aggregation are essential for primary hemostasis but are also critically involved in the development of acute arterial thrombotic occlusion at regions of atherosclerotic plaque rupture after contact to subendothelial collagen, the major pathophysiological mechanism underlying myocardial infarction and ischemic stroke.1 Platelet activation caused by exposed collagen involves glycoprotein VI (GPVI) and integrin αIIbβ3.2 Downstream of these platelet surface receptors, collagen induces intracellular signaling cascades involving tyrosine kinases as well as small GTPases, resulting in an activation of phospholipase Cγ2 (PLCγ2) followed by an increase of cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]i).3,5 PLCγ2-dependent increase of cytosolic [Ca$^{2+}$], is critically important for platelet activation and consecutive occlusive thrombus formation by triggering inositol-1,4,5-trisphosphate (IP3)–mediated rapid Ca$^{2+}$ release from intracellular stores via binding to IP3 receptors with subsequent activation of extracellular Ca$^{2+}$ influx.6 A critical component of intracellular platelet signaling pathways downstream of the collagen receptors integrin αIIbβ3 and GPVI that controls platelet activation is the phosphoinositide 3-kinase (PI3K) signaling cascade.7,8 PI3K, as well as its downstream target protein kinase B (Akt), has previously been shown to facilitate activation-dependent Ca$^{2+}$ influx into platelets.12,14 To discriminate...
GPVI-dependent intracellular signaling induced by collagen from signaling processes downstream of other collagen receptors as integrin \(\alpha_\beta\), collagen-related peptide (CRP) or the snake venom convulxin can be used as platelet agonists targeting the platelet collagen receptor GPVI.\(^{15,16}\)

Phosphoinositide-dependent protein kinase 1 (PDK1) is a member of the AGC serine/threonine kinase family that is activated downstream of PI3K and regulates several further AGC kinases, including Akt and serum- and glucocorticoid-inducible kinase 1 (SGK1) by phosphorylating the activation loops of these enzymes.\(^{17}\) Several agonists activate platelet PI3K, resulting in generation of membrane-localized phosphoinositides phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate.\(^{18}\) Driven by these phosphoinositides, Akt is recruited to the membrane, enabling its phosphorylation by PDK1 at Thr\(^{308}\) after a major conformational change.\(^{19}\) PDK1/AGC kinase signaling pathways regulate diverse cellular processes, such as cell survival, cell proliferation, metabolism, neuronal response, vascular remodeling, and heart failure.\(^{17,20,21}\)

Recently, PDK1 has been shown to play an important role in thrombin-induced platelet aggregation and clot retraction by regulating the integrin \(\alpha_\beta_3\), outside-in signaling via Akt-dependent inhibition of glycogen synthase kinase-3\(\beta\).\(^{22}\) Moreover, PDK1 inhibitor BX795 was found to reduce efficiently Akt phosphorylation and functional activation of human platelets.\(^{23}\) In nucleated cells, PDK1 regulates activation-dependent intracellular Ca\(^{2+}\) increase and actin cytoskeleton dynamics,\(^{24,25}\) processes both essential for platelet adhesion and thrombus formation after contact to collagen. Nevertheless, neither the role of PDK1 in Ca\(^{2+}\) influx nor the influence of PDK1 on Ca\(^{2+}\)-driven platelet activation after contact to collagen have been investigated to date. Genetic deletion of PDK1 causes embryonic lethality.\(^{20,21,26}\) To circumvent prenatal death and be able to investigate a platelet-specific effect of PDK1 on arterial thrombotic vascular occlusion in vivo, mice expressing PDK1 alleles flanked with the \(loxP-Cre\) excision sequence (\(pdk1^{fl/fl}\)) were used to achieve cell-specific deletion of PDK1.\(^{21,27}\)

In the present study, we aimed to identify the role of PDK1 in collagen-triggered Ca\(^{2+}\) signaling and the underlying mechanisms in PDK1-driven platelet activation in response to collagen. By using several murine in vivo models, we further investigated the role of PDK1 for in vivo arterial thrombotic occlusion and ischemic stroke.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

For examination of the role of PDK1 in platelet Ca\(^{2+}\) signaling, as well as platelet-triggered thrombotic arterial occlusion and development of ischemic stroke, we generated platelet-specific PDK1 knockout mice (\(pdk1^{fl/fl}\)) in combinaison with \(p4f4^{-}\). Reverse transcription polymerase chain reaction analysis and immunoblotting revealed the substantial loss of PDK1 expression in \(pdk1^{−/−}\) platelets compared with \(pdk1^{fl/fl}\) platelets on transcript and protein level, whereas PDK1 expression in other tissues (as shown in the kidney) was unaffected (Figure 1A and 1B). Fluorescence-activated cell sort

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Akt</td>
<td>protein kinase B</td>
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<tr>
<td>CRP</td>
<td>collagen-related peptide</td>
</tr>
<tr>
<td>GPVI</td>
<td>glycoprotein VI</td>
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<tr>
<td>IP3</td>
<td>inositol-1,4,5-trisphosphate</td>
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<tr>
<td>PDK1</td>
<td>phosphoinositide-dependent protein kinase 1</td>
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<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
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<tr>
<td>PLC(\gamma\2</td>
<td>phospholipase C(\gamma\2</td>
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<td>SGK1</td>
<td>serum- and glucocorticoid-inducible kinase 1</td>
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**Figure 1.** Characterization of phosphoinositide-dependent protein kinase 1 (PDK1)–defective platelets and PDK1–dependent protein kinase B (Akt) phosphorylation at Thr\(^{308}\) on collagen-triggered platelet activation. **A**, Representative image (bottom) and arithmetic means±SEM (n=4; top) of PDK1 transcript levels in platelets and kidneys from \(pdk1^{fl/fl}\) (\(pdk1^{fl/fl}\!/\!p4f4^{-}\!/\!Cre^{-}\)) and \(pdk1^{−/−}\) (\(pdk1^{fl/fl}\!/\!p4f4^{-}\!/\!Cre^{-}\)) mice. **B**, Representative Western blot (bottom) and arithmetic means±SEM (n=4; top) of PDK1 protein levels in platelets and kidneys from \(pdk1^{fl/fl}\) (\(pdk1^{fl/fl}\!/\!p4f4^{-}\!/\!Cre^{-}\)) and \(pdk1^{−/−}\) (\(pdk1^{fl/fl}\!/\!p4f4^{-}\!/\!Cre^{-}\)) mice. **C–E**, Akt phosphorylation (at Thr\(^{308}\)) in \(pdk1^{fl/fl}\) and \(pdk1^{−/−}\) platelets after stimulation with collagen-related peptide (CRP; 1 \(\mu\)g/mL). Total Akt was used as loading control. Arithmetic means±SEM (n=4). **C**, Akt phosphorylation (at Thr\(^{308}\)) in \(pdk1^{fl/fl}\) and \(pdk1^{−/−}\) platelets after stimulation with convulxin (CVX; 0.25 \(\mu\)g/mL). Total Akt was used as loading control. Arithmetic means±SEM (n=4). **D**, Akt phosphorylation (at Thr\(^{308}\)) in \(pdk1^{fl/fl}\) and \(pdk1^{−/−}\) platelets after stimulation with CRP (1 \(\mu\)g/mL). Total Akt was used as loading control. Arithmetic means±SEM (n=5). *P<0.05 and **P<0.01 indicate statistically significant differences.
analysis revealed comparable surface expression levels of platelet surface receptors engaged in platelet adhesion to collagen as integrin α<sub>IIb</sub>β<sub>3</sub>, integrin α<sub>IIb</sub>β<sub>3</sub>, GPVI, and glycoprotein Ib-IX-V complex in pdk1<sup>fl/fl</sup> and pdk1<sup>−/−</sup> platelets (Table). As examined by immunoblotting and shown in Figure 1C and 1D, the PDK1 downstream target Akt is strongly phosphorylated in pdk1<sup>−/−</sup> platelets after stimulation with the GPVI agonists CRP or convulxin. In PDK1-deficient platelets, phosphorylation of Akt at Thr<sup>308</sup> initiating Akt activation was totally abolished (Figure 1C and 1D). GPVI-dependent activation of Akt was still significantly reduced in pdk1<sup>−/−</sup> as compared with pdk1<sup>fl/fl</sup> platelets in the presence of indomethacin (cyclooxygenase inhibition) and apyrase (hydrolysis of ADP) as inhibitors of second-wave platelet activation after stimulation with CRP (Figure 1E).

Because subendothelial collagen is a crucial trigger of platelet adhesion and aggregation followed by occlusive thrombus formation after plaque rupture in myocardial infarction or ischemic stroke, we examined the role of PDK1 in intracellular platelet signaling and PDK1-dependent mechanisms of platelet activation on stimulation with collagen/CRP. To elucidate the impact of PDK1 on platelet secretion, the activation-dependent release of platelet alpha (P-selectin exposure) and dense (ATP release) granules was quantified by fluorescence-activated cell sorter and luminescence analysis. As illustrated in Figure 2A and 2B, pdk1<sup>−/−</sup> platelets displayed significantly reduced secretion of alpha and dense granules after stimulation with low and intermediate concentrations of CRP. According to electron microscopy and immunoblotting of P-selectin expression in resting platelets, number and content of alpha and dense granules were comparable in pdk1<sup>fl/fl</sup> and pdk1<sup>−/−</sup> platelets (Figure IA and IB in the online-only Data Supplement). Moreover, integrin α<sub>IIb</sub>β<sub>3</sub>, activation required for fibrinogen binding facilitating platelet aggregation was significantly diminished in pdk1<sup>−/−</sup> platelets compared with pdk1<sup>fl/fl</sup> platelets after stimulation with CRP (Figure 2C). Light transmission aggregometry was used to explore whether impaired secretion and integrin α<sub>IIb</sub>β<sub>3</sub>, activation would translate into reduced platelet aggregation. After stimulation with increasing concentration of CRP, aggregation of pdk1<sup>−/−</sup> platelets was found to be significantly blunted as compared with aggregation of platelets from pdk1<sup>fl/fl</sup> mice (Figure 2D). Defective degranulation, integrin α<sub>IIb</sub>β<sub>3</sub>, activation, and aggregation in pdk1<sup>−/−</sup> platelets were overcome by high concentrations of CRP.

A further series of experiments examined the consequences of impaired activation of pdk1<sup>−/−</sup> platelets for platelet adhesion to collagen and thrombus formation at high arterial shear rates (1700 s<sup>−1</sup>). While pdk1<sup>fl/fl</sup> platelets rapidly adhered to collagen-coated surfaces and formed stable thrombi, thrombus formation by pdk1<sup>−/−</sup> platelets was significantly impaired because the thrombus surface coverage was reduced by ≈40% (Figure 2E). To investigate whether abrogated adhesion and thrombus formation in pdk1<sup>−/−</sup> mice is a result of impaired second-wave signaling via purinergic receptors or because of diminished thromboxane A<sub>2</sub>, we repeated the flow chamber experiments in the presence of apyrase (2 U/mL) and indomethacin (10 μmol/L) as described previously. As illustrated in Figure 2E, in the presence of apyrase and indomethacin, thrombus formation in vitro under high arterial shear rates was still significantly reduced in pdk1<sup>−/−</sup> platelets as compared with pdk1<sup>fl/fl</sup> platelets. Furthermore, the significance of reduction in pdk1<sup>−/−</sup> platelets was even more pronounced in the presence (P=0.02) than in the absence of second-wave inhibitors (P=0.04).

Because PDK1 has been reported to modify actin cytoskeleton reorganization and to act as potential regulator of Rac1 in neuronal cells, we elucidated the role of PDK1-dependent signaling in collagen-induced lamellipodia formation and platelet spreading. According to Rac1 activation assay (Figure 3A), stimulation of pdk1<sup>fl/fl</sup> platelets with increasing concentrations of CRP resulted in a rapid and strong increase of Rac1-GTP binding, illustrating Rac1 activation, an effect significantly blunted in pdk1<sup>−/−</sup> platelets, indicating that PDK1/Akt signaling is involved in platelet Rac1 activation downstream of GPVI after stimulation with CRP. Defective Rac1 activation in pdk1<sup>−/−</sup> platelets was paralleled by significantly impaired lamellipodia formation and spreading of pdk1<sup>−/−</sup> platelets on fibrinogen-coated surfaces after stimulation with CRP after 5, 30, and 45 minutes (Figure 3C and 3E). According to scanning electron microscopy, PDK1 is critically involved in GPVI-dependent platelet adhesion and spreading because PDK1-deficient platelets display defective spreading on fibrinogen after stimulation with 5 μg/mL CRP (Figure 3B). Surface area of spread platelets on fibrinogen was significantly diminished in pdk1<sup>−/−</sup> platelets as compared with pdk1<sup>fl/fl</sup> platelets after stimulation with CRP for 30 minutes or 45 minutes (Figure 3D).

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<th>Table. Arithmetic Means±SEM (n=6) of Glycoprotein Surface Expression in pdk1&lt;sup&gt;fl/fl&lt;/sup&gt; and pdk1&lt;sup&gt;−/−&lt;/sup&gt; Platelets</th>
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<td></td>
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<tr>
<td>GPⅠbα</td>
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<td>GPⅠbβ</td>
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<td>GPⅠV</td>
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<td>GPIⅢ</td>
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<td>Integ. α&lt;sub&gt;IIb&lt;/sub&gt;β&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>Integ. β&lt;sub&gt;1&lt;/sub&gt;</td>
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GPⅠb indicates glycoprotein Ib; GPⅠb, glycoprotein Ib; GPⅠV, glycoprotein V; GPⅠV, glycoprotein V; GPIⅢ, glycoprotein IX; and PDK1, phosphoinositide-dependent protein kinase 1.
similar in \( \text{pdk1}^{+/+} \) and \( \text{pdk1}^{-/-} \) platelets. In presence as well as in absence of extracellular Ca\(^{2+}\), \( \text{pdk1}^{-/-} \) platelets display a significantly diminished increase of \([\text{Ca}^{2+}]_{i}\) in response to stimulation with CRP (5 \( \mu \)g/mL) or convulxin (1 \( \mu \)g/mL), pointing to an impaired intracellular regulation of cytosolic Ca\(^{2+}\) activity in PDK1-deficient platelets. To test whether the decreased intracellular Ca\(^{2+}\) release could have been because of a defective PLC\(\gamma\)-2-triggered IP\(_3\) production, we investigated IP\(_3\) production, reflecting the levels of the unstable IP\(_3\), in \( \text{pdk1}^{-/-} \) platelets in presence or absence of the PLC inhibitor U73122. As shown in Figure 4C, stimulation with CRP (2.5 \( \mu \)g/mL) or convulxin (500 ng/mL) was followed by a significant increase of IP\(_3\) production, which was significantly less pronounced in \( \text{pdk1}^{-/-} \) platelets than in \( \text{pdk1}^{+/+} \) platelets. Addition of PLC inhibitor U73122 (10 \( \mu \)mol/L) abolished the increase of IP\(_3\) after stimulation with CRP and dissipated the differences between \( \text{pdk1}^{+/+} \) and \( \text{pdk1}^{-/-} \) platelets (Figure 4D). However, tyrosine phosphorylation of PLC\(\gamma\)-2 on GPVI stimulation was not significantly different in \( \text{pdk1}^{-/-} \) platelets compared with \( \text{pdk1}^{+/+} \) platelets (Figure II in the online-only Data Supplement).
To assess the significance of PDK1 for arterial thrombus formation in vivo, time to occlusion of mesenteric arterioles because of thrombus formation after FeCl₃-induced injury has been analyzed. As displayed in Figure 5A and 5B, arterial thrombotic occlusion was significantly delayed and decreased in pdk₁⁻⁻ mice after FeCl₃-triggered vascular injury of mesenteric arterioles. Nearly 60% of the pdk₁⁻⁻ mice failed to form an occlusive thrombus within 30 minutes, whereas in wild-type mice, the mean time to thrombotic vascular occlusion was some 14 minutes. To test whether the defect in pdk₁⁻⁻ platelets was paralleled by impaired hemostasis, we measured tail bleeding time in PDK1 floxed mice. As depicted in Figure 5C, pdk₁⁻⁻ mice showed a minor bleeding tendency compared with pdk₁⁺⁺ mice (397 s versus 275 s; P<0.01).

In a next step, we explored the impact of platelet PDK1 on development and outcome after ischemic stroke. To this end, mice with a platelet-specific knockout of PDK1 were studied in the middle cerebral ischemia occlusion model. As shown in Figure 5D and 5E, brain infarct volumes were significantly reduced in pdk₁⁻⁻ mice compared with infarct volumes in littermate wild-type mice (pdk₁⁺⁺; 65.85 mm³ versus 100.4 mm³; P<0.01). To confirm the protective effect of PDK1...
deficiency on ischemic brain infarct development, magnetic resonance imaging on living mice was performed. As illustrated by serial T2-weighted magnetic resonance imaging, hyperintense, ischemic brain infarcts were markedly reduced in pdk1−/− mice compared with wild-type littermates (pdk1 fl/fl) after transient middle cerebral artery occlusion, a protective effect that was sustained over 7 days (Figure 5E). Pdk1−/− mice further showed a significantly improved outcome because PDK1 deficiency resulted in an increased survival 7 days after transient middle cerebral artery occlusion (50% versus 33%; \( P < 0.05 \)). Markedly, no intracranial hemorrhages were detected in pdk1−/− mice after transient middle cerebral artery occlusion (Figure 5E).

**Discussion**

Platelets play a crucial role in the pathogenesis of arterial thrombosis, and the mechanisms regulating adhesive functions of platelets are thus of pivotal importance for occlusive cardiovascular diseases as myocardial infarction and ischemic stroke.5,31,32 Acute myocardial infarction and ischemic stroke represent the leading causes of death and permanent disability in industrialized countries, despite ongoing improvements in antithrombotic therapy.1,33 By understanding the multifaceted intracellular signaling involved in platelet activation underlying hemostasis as well as arterial thrombosis, new approaches can be tailored to selectively inhibit pathways most relevant to pathological thrombus formation.5 Subendothelial collagen exposed to platelets after rupture of atherosclerotic plaques is the main trigger of platelet activation and acute arterial thrombotic occlusion. PI3K-dependent signaling downstream of the collagen receptor GPVI is essential for platelet activation.34 The major findings of the present study are the following: (1) PDK1 becomes activated by GPVI agonists CRP and convulxin; (2) PDK1 determines collagen-dependent increase in platelets via phospholipase C\(_2\)-induced inositol-1,4,5-trisphosphate (IP\(_3\)) production. A. Representative tracings (right) of Fura-2-fluorescence reflecting cytosolic Ca\(^{2+}\) concentration [Ca\(^{2+}\)]\(_i\) and arithmetic means (left) of maximal \(\Delta[Ca^{2+}]_i\)±SEM (n=5–8) of pdk1\(^{1/2}\) (black tracings and bars) and pdk1−/− (gray tracings and bars) platelets before and after stimulation with collagen-related peptide (CRP; 5 \(\mu\)g/mL) or convulxin (CVX; 1 \(\mu\)g/mL) in the absence (0.5 mmol/L EGTA) of extracellular Ca\(^{2+}\). B. Representative tracings (right) of Fura-2 fluorescence reflecting cytosolic Ca\(^{2+}\) concentration [Ca\(^{2+}\)]\(_i\) and arithmetic means (left) of maximal \(\Delta[Ca^{2+}]_i\)±SEM (n=5–8) of pdk1\(^{1/2}\) (black tracings and bars) and pdk1−/− (gray tracings and bars) platelets before and after stimulation with CRP (5 \(\mu\)g/mL) or CVX (1 \(\mu\)g/mL) in the presence (1 mmol/L Ca\(^{2+}\)) of extracellular Ca\(^{2+}\). C. Quantification of produced IP\(_3\)-specific metabolite IP\(_1\), on stimulation with CRP (2.5 \(\mu\)g/mL) or CVX (0.5 \(\mu\)g/mL) using ELISA assay. Arithmetic means±SEM (n=8) of IP\(_3\) concentrations (nM) are shown. D. Quantification of produced IP\(_3\)-specific metabolite IP\(_1\), on stimulation with CRP (1 \(\mu\)g/mL) in the presence of phospholipase (PLC) inhibitor U73122 (PLC inh; 10 \(\mu\)mol/L) using ELISA assay. DMSO was used as solvent control (vehicle). Arithmetic means±SEM (n=8–13) of IP\(_3\) concentrations (nM) are shown. Significant results are indicated by \(* (P<0.05)\) and \(** (P<0.01)\), respectively.
of [Ca\(^{2+}\)]\(_i\) in platelets via regulation of PLC\(\gamma\)2-dependent IP\(_3\) production downstream of collagen receptor GPVI; (3) PDK1 is essential for platelet activation (secretion and aggregation) and adhesion on stimulation with collagen/CRP; (4) PDK1 is crucial to CRP-triggered Rac1 activation and lamellipodia formation; and (5) PDK1 is a potent regulator of arterial thrombosis and ischemic stroke in vivo.

Our data suggest that PDK1 is an important regulator of Akt activation via phosphorylation of Thr\(^{308}\) after induction of platelet GPVI-dependent signaling. PDK1 links PI3K to PLC\(\gamma\)2-dependent IP\(_3\) production and intracellular Ca\(^{2+}\) release in response to collagen-triggered platelet activation via collagen receptor GPVI. PI3K has already been identified to be involved in collagen-induced enhancement of PLC\(\gamma\)2 and intracellular Ca\(^{2+}\) release downstream of GPVI because disrupting PI3K\(\alpha\) or PI3K\(\beta\)-dependent signaling resulted in suppressed collagen-induced Ca\(^{2+}\) mobilization and IP\(_3\) production.\(^{10,14,34,35}\) In our study, we used CRP and the snake venom convulxin as platelet agonists that target GPVI and induce GPVI-dependent signaling without activating the other main collagen receptor integrin \(\alpha\_\beta\_3\).\(^{36}\) Because platelet Ca\(^{2+}\) increase and activation was profoundly affected in PDK1-deficient platelets after stimulation with GPVI-specific agonist CRP (and also convulxin), we conclude that PDK1 is critically involved in collagen-induced Ca\(^{2+}\)-dependent platelet activation downstream of the collagen receptor GPVI. SGK1, beside Akt, one of the major downstream effectors of PDK1,\(^{37}\) has recently been identified to regulate platelet Orai1-dependent extracellular Ca\(^{2+}\) influx in response to GPVI activation.\(^{12}\) But neither SGK1 nor store-operated Ca\(^{2+}\) channel moieties Orai1 were differentially regulated in \(pdk1\^−/−\) platelets (data not shown). Further, in contrast to \(sgk1\^−/−\) platelets, which have a defective extracellular Ca\(^{2+}\) entry while intracellular Ca\(^{2+}\) release is not affected,\(^{12}\) \(pdk1\^−/−\) platelets apparently displayed a significantly reduced release of Ca\(^{2+}\) from intracellular stores, pointing to a role of PDK1 in activation-dependent Ca\(^{2+}\) increase, which is different from that of SGK1.

PLC\(\gamma\)2, and partially PLC\(\gamma\)1, plays a decisive role in platelet IP\(_3\) production and Ca\(^{2+}\)-driven platelet activation by collagen.\(^{38}\) IP\(_3\) releases Ca\(^{2+}\) from intracellular stores in platelets by directly activating IP\(_3\) receptors in platelet endoplasmatic reticulum.\(^{39}\) In metastatic human breast cancer cell lines, PDK1 regulates PLC\(\gamma\) with consecutive accumulation of inositol phosphatases and intracellular Ca\(^{2+}\) mobilization via tyrosine phosphorylation.\(^{40}\) Similar to \(plc\_\gamma2\^−/−\) platelets,\(^{38,40,41}\) PDK1-deficient platelets display reduced intracellular Ca\(^{2+}\) release and impaired adhesion to collagen under flow conditions, as well as abolished thrombus formation in vivo after vascular injury of mesenteric arterioles in mice. However, the extent of defects in platelet functions in response to collagen-induced activation found in \(pdk1\^−/−\) platelets were not as strong as found in \(plc\_\gamma2\^−/−\) platelets, an observation pointing...
to PDK1-independent pathways leading to PLCγ activation. Beside GPVI-dependent increase of \([\text{Ca}^{2+}]_i\), PDK1 may be involved in secondary signaling amplifications via autocrine-released ADP on stimulation with collagen because second-wave mediator ADP was shown to play an important role in GPVI-dependent thrombus formation.42,43 By inhibition of ADP-induced activation of purinergic receptors, as well as cyclooxygenase-triggered thromboxane A2 synthesis, we could identify PDK1 as a direct effector of GPVI activation in platelets because Akt phosphorylation and in vitro thrombus formation were still significantly impaired, even more pronounced in the presence of indomethacin and apyrase. These results are in line with previous studies reporting that second-wave mediators ADP and thromboxane A2 have a much greater feedback role in platelet activation by collagen downstream of integrin αβ3 than downstream of the platelet collagen receptor GPVI.25 Nevertheless, impaired secretion in \(\text{pdk1}^{-/-}\) mice downstream of GPVI results in a reduced paracrine activation because of diminished ADP secretion from dense granules (as reflected by reduced ATP release).

As shown in several previous studies, the small GTPase Rac1 is an essential regulator of PLCγ2 activity.44,45 Platelets lacking Rac1 display defective GPVI-dependent IP3 production and intracellular Ca2+ mobilization, leading to impaired adhesion, secretion, aggregation, and thrombus formation on collagen.45,46 According to the results of this study, platelet Rac1 is activated by PDK1 in response to the GPVI agonist CRP. These findings are in line with broad evidence from other cells and diseases showing that Rac1 is activated by PI3K/Akt-dependent signaling47 and with a recent study identifying Rac1 as a downstream target of PDK1.29 As suggested by comparison with platelets isolated from \(\text{racf1}^{-/-}\) mice,45 PDK1 may activate PLCγ2 via Rac1 without affecting PLCγ2 phosphorylation because \(\text{pdk1}^{-/-}\) platelets display an inappropriate PLCγ2 activation, despite normal enzyme tyrosine phosphorylation.

In other cell types, PDK1 has been reported to play an important role in the regulation of actin cytoskeleton dynamics and focal adhesion.23,48,49 Recently, Chen et al examined the role of PDK1 in platelet outside-in signaling involving integrin αIIbβ3-mediated platelet adhesion to fibrinogen.22 Sustained Rac1 activation is required for lamellipodia formation underlying platelet shape change and spreading.46,50 as well as as platelet-triggered thrombus formation in vivo.51 Indeed, \(\text{pdk1}^{-/-}\) platelets show impaired spreading on fibrinogen on stimulation with CRP. Comparable to Rac1-deficient mice,45,46 we observed blunted GPVI-mediated platelet activation and adhesion50 on stimulation with CRP and sufficient protection against ischemic stroke with prolonged bleeding times in \(\text{pdk1}^{-/-}\) mice. Beside its activation downstream of PI3K/PDK1 signaling, Rac1 triggers PI3K-dependent signaling, resulting in a positive feedback loop of Rac1 regulation.10,16,46 Rac1 activity was even more reduced in resting \(\text{pdk1}^{-/-}\) platelets, pointing to a kind of nonconditional connection between PDK1 and its downstream effector Rac1 in unstimulated platelets which may be bypassed after GPVI-dependent platelet activation, independently of the PI3K/PDK1 signaling pathway. Platelet spreading defects only partially reflect the markedly reduced Rac1 activity, which results from the fact that beside Rac1, many other players in cytoskeletal organization, which are not under control of PDK1, play a role in platelet spreading.

Ischemic stroke is the third leading cause of death, but mechanisms underlying thrombotic occlusions in the brain are poorly understood.52 In the present study, we could identify PDK1 as a novel and promising target of antiplatelet strategy to prevent ischemic stroke after thrombotic vascular occlusion. Emerging evidence indicates an important link between thrombotic and inflammatory pathways (thrombo-inflammation) in the pathophysiology of ischemic stroke.52 Beside forming occlusive arterial thrombi by platelet activation after contact to exposed collagen, GPVI-dependent signaling also contributes to secretion of platelet-derived inflammatory cytokines, mediating cerebrovascular inflammation in animal models of cerebral ischemia.33,52

\(\text{pdk1}^{-/-}\) platelets are still able to aggregate and show no signs of spontaneous bleeding or hemorrhage, suggesting that other PDK1-independent signaling pathways can compensate for these deficiencies. Nevertheless, beside defective thrombus formation and the protection against ischemic stroke in platelet-specific PDK1-deficient mice, \(\text{pdk1}^{-/-}\) seems to be significantly involved in platelet-dependent primary hemostasis because \(\text{pdk1}^{-/-}\) mice showed a mild but significant increase of tail bleeding time without affecting surface expression of platelet glycoproteins critical to hemostasis. Similar to GPVI-deficient mice, thrombosis is impacted to a larger extent than hemostasis in \(\text{pdk1}^{-/-}\) mice. It has been reported that GPVI-deficient mice are protected against thrombus formation in vitro and in vivo, but do not show severely increased tail bleeding time in vivo.53,54 We, therefore, hypothesize that increased bleeding time in \(\text{pdk1}^{-/-}\) mice may be a result of additionally impaired GPCR-dependent signaling in these mice.

In conclusion, the present study unravels PDK1 as a central regulator of intracellular Ca2+ increase in platelets, platelet activation, and aggregability after stimulation with collagen. Thus, PDK1 is a critical player in platelet-triggered thrombus formation and ischemic stroke.

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**Disclosures**

None.

**References**

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