IMPAIRED THROMBIN-INDUCED PLATELET ACTIVATION AND THROMBUS FORMATION IN MICE LACKING THE Ca²⁺-DEPENDENT TYROSINE KINASE Pyk2

Ilaria Canobbio¹*, Lina Cipolla²*, Alessandra Consonni¹, Stefania Momi³, Gianni Guidetti¹, Barbara Oliviero⁴, Marco Falasca⁵, Mitsuhiko Okigaki⁶, Cesare Balduini¹, Paolo Gresele⁹, Mauro Torti¹

¹Department of Biology and Biotechnology, Division of Biochemistry, University of Pavia, Italy
²Department of Molecular Medicine, University of Pavia, Italy
³Department of Internal Medicine, University of Perugia, Italy
⁴Department of Infectious Diseases, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
⁵Blizard Institute, Queen Mary University of London, UK
⁶Department of Cardiovascular Medicine, Kyoto Prefectural University of Medicine, Japan

Running head: The role of Pyk2 in platelet activation

* These authors equally contributed to the work

Corresponding author: Mauro Torti, Department of Biology and Biotechnology, Division of Biochemistry, University of Pavia, via Bassi 21, 27100 Pavia. Phone: *39-0382-987238; FAX: 0382-987240; E-mail: mtorti@unipv.it

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SUMMARY

In this study, we used a knockout murine model to analyze the contribution of the Ca\textsuperscript{2+}-dependent focal adhesion kinase Pyk2 in platelet activation and thrombus formation \textit{in vivo}. We found that Pyk2 knockout mice had a tail bleeding time slightly increased as compared with wild type littermates. Moreover, in an \textit{in vivo} model of femoral artery thrombosis, the time to arterial occlusion was significantly prolonged in mice lacking Pyk2. Pyk2-deficient mice were also significantly protected from collagen plus epinephrine-induced pulmonary thromboembolism. \textit{Ex vivo} aggregation of Pyk2-deficient platelets was normal upon stimulation of GPVI, but it was significantly reduced in response to PAR4-activating peptide, low doses of thrombin, or U46619. Defective platelet aggregation was accompanied by impaired inside-out activation of integrin α\textsubscript{II}β\textsubscript{3}, and fibrinogen binding. Granule secretion was only slightly reduced in the absence of Pyk2, while a marked inhibition of thrombin-induced TxA\textsubscript{2} production was observed, and was found to be responsible for the defective aggregation. Moreover, we demonstrated that Pyk2 is implicated in the signaling pathway for cPLA\textsubscript{2} phosphorylation through p38MAPK. These results show the importance of the focal adhesion kinase Pyk2 downstream of G-protein-coupled receptors to support platelet aggregation and thrombus formation.
INTRODUCTION

Pyk2, also known as RAFTK or CADβ, is a non receptor tyrosine kinase highly homologous to the focal adhesion kinase FAK, and predominantly expressed in the central nervous system and in hematopoietic cells.\textsuperscript{1-3} Like FAK, Pyk2 does not possess SH2 or SH3 domains, but has a centrally located catalytic domain flanked by a N-terminal FERM domain and a C-terminal focal adhesion targeting FAT domain. Pyk2 can be tyrosine phosphorylated and activated by a variety of different cellular stimuli, including cytokines, growth factors, agonists of G-protein-coupled receptors (GPCRs), integrin ligands, stress stimuli.\textsuperscript{3-6} Typically, Pyk2 activation is mediated by both Src kinases- and cytosolic Ca\textsuperscript{2+}-dependent pathways. Src kinases phosphorylate Pyk2 at Tyr579, Tyr580, and Tyr881, increasing the catalytic activity of the kinase and promoting autophosphorylation on Tyr402 in the FERM domain.\textsuperscript{5,6} Even in the absence of Src-mediated phosphorylation, Pyk2 can be activated upon increase of the cytosolic Ca\textsuperscript{2+} concentration.\textsuperscript{4-7} It has been shown that Ca\textsuperscript{2+} and calmodulin bind to the N-terminal FERM domain of Pyk2, triggering Pyk2 dimerization, activation, and autophosphorylation at Tyr402.\textsuperscript{8} Phosphorylated Tyr402 represents, in turn, a binding site for the SH2 domain of Src.\textsuperscript{5,6} Therefore Pyk2 is a kinase that can link Ca\textsuperscript{2+}-based signaling pathways to protein tyrosine phosphorylation.

Some observations reported over the past two decades have implicated Pyk2 in platelet activation. Pyk2 is highly expressed in megakaryocytes and platelets, and is rapidly phosphorylated upon stimulation with several platelet agonists, including thrombin, collagen, ADP, estrogens, and von Willebrand factor.\textsuperscript{9-13} Phosphorylation of Pyk2 downstream of integrin αIIbβ3 and α2β1 engagement has also been reported.\textsuperscript{10,14} In thrombin- and von Willebrand factor-activated platelets, Pyk2 interacts with the actin-based cytoskeleton,\textsuperscript{9,11} and other reports have documented its association with the phosphatidylinositol 3-kinase (PI3K), Cas, Shc and Hic-5.\textsuperscript{15-18} Thrombin-induced Pyk2 activation was found to be regulated by Ca\textsuperscript{2+} and protein kinase C, as well as by cytoskeleton reorganization.\textsuperscript{9,10,18} Because of such a limited amount of available information, the role of Pyk2 in platelet function still remains unknown.

Pyk2 knockout mice have been generated, and have provided important clues on the function of this kinase in inflammation, atherosclerosis and angiogenesis. For instance, Pyk2 has been demonstrated to play a crucial role in macrophage adhesion and migration, by regulating multiple signaling events triggered by chemokine stimulation, including activation of Rho GTPase, phospholipase C (PLC), and PI3K.\textsuperscript{19} Moreover, endothelial cell migration
and tube formation promoted by vascular endothelial growth factor was strongly impaired in the absence of Pyk2.\textsuperscript{20} A recent report also documented that Pyk2 is implicated in ROS generation and in ROS-mediated proinflammatory events involved in atherosclerosis.\textsuperscript{21} Altogether, these observations clearly point to a relevant role for Pyk2 in the cardiovascular system.

In the present study, Pyk2 knockout mice were used in order to unravel the function of this kinase in platelet activation and \textit{in vivo} thrombus formation. We show that Pyk2 is an important mediator of thrombin-induced platelet aggregation. Moreover, we demonstrate that Pyk2-deficient mice are protected against arterial thrombosis and pulmonary thromboembolism. These results show an essential role of Pyk2 in the signaling pathways regulating the function of platelets in haemostasis and thrombosis.
MATERIALS AND METHODS

Materials.

Thrombin, U46619, ADP were from Sigma (Milan, Italy). Convulxin was provided by Dr. K. J. Clemetson (Theodor Kocher Institute, University of Berne, Switzerland). AYPGKF was synthesized by PRIMM (Milan, Italy). Collagen was from Hormon-Chemie (München, Germany). Epinephrine and arachidonic acid were from Mascia Brunelli (Milan, Italy). FITC-conjugated anti-mouse P-selectin (M130-1) and PE-JON/A (M023-2) were from Emfret analytics (Wurzburg, Germany). VX-702 was from Tocris (Space Import Export, Milan, Italy). FITC-labeled fibrinogen was from Molecular Probes (Milan, Italy). Anti-phospho-cPLA2(Ser505), anti-phosphoERK1/2(Thr202/Thr204), and anti phospho-p38MAPK antibodies were from Cell Signaling Technology (Euroclone, Pero, Italy). The rabbit polyclonal antibodies against Pyk2 (N-19), and against FAK (A17), against p38MAPK (C-20), and against ERK (C-14), as well as the monoclonal antibody anti-tubulin (DM1A), were from Santa Cruz Biotechnology (Tebu-Bio, Magenta, Italy). Appropriate peroxidase-conjugated anti-IgG antibodies were from Bio-Rad (Milan, Italy). SB203580 was from Calbiochem (Merck Millipore, Milan, Italy). Thromboxane B2 EIA kit was from DRG Diagnostic (Milan, Italy). Pyk2 knockout (KO) mice generation was described elsewhere.19 All the procedures involving the use of C57BL/6 (WT) and Pyk2 KO mice were approved by the Ethics Committee of the University of Pavia, by the Committee on Ethics of Animal Experiments of the University of Perugia and by the Italian Ministry of Public Health (Authorization n° 230/2010-231/2010-B and 232/2010-B).

Preparation of washed mouse platelets.

Blood was withdrawn from the abdominal vena cava of anesthetized animals in syringes containing ACD/3.8% sodium citrate (2:1) as anticoagulant. Anticoagulated blood was diluted with HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.9 mM KCl, 12 mM NaHCO₃, pH 7.4) up to 2 ml and centrifuged for 7 minutes at 180g to obtain platelet-rich-plasma (PRP). PRP was then transferred to new tubes and the remaining red blood cells were diluted with HEPES buffer to a final volume of 2 ml and centrifuged again at 180g for 7 minutes. The upper phase was added to the previously collected PRP, and 0.02 U/ml apyrase, and 1 μM PGE₁ were added before centrifugation at 550g for 10 minutes. The supernatant PPP was removed, and the platelet pellet was resuspended in 500 μl of HEPES buffer.
Platelet and white cell count in whole blood.

Blood was collected by the inferior vena cava from mice under ether anesthesia and anticoagulated with 1/10 volume of tripotassium EDTA. After mixing, platelets were counted optically, by the Brecher-Cronkite method, by an operator unaware of the experimental groups.22,23

Immunoblotting analysis.

Platelet samples (0.1 ml, 5×10^8 platelets/ml) were incubated at 37°C and stimulated with different doses of thrombin or treated with an equivalent volume of HEPES buffer for increasing times. Reactions were stopped by the addition of 0.05 ml of SDS-sample buffer 3X (37.5 mM Tris, 288 mM glycine, pH 8.3, 6% SDS, 1.5% DTT, 30% glycerol, 0.03% bromophenol blue). Samples were heated at 95°C for 5 minutes, and proteins from aliquots of identical volume were separated by SDS-PAGE, transferred to PVDF membranes and probed by immunoblotting with the antibodies indicated in the text and in the figure legends at a 1:500 dilution, as previously described.24 Images of reactive bands were acquired using a Chemidoc XRS apparatus (Bio-Rad, Milan, Italy), and quantification of band intensity was performed using the QuantityOne software.

Platelet aggregation.

Washed platelets from wild type and Pyk2 KO mice (0.25 ml, 3×10^8 platelets/ml) were stimulated under constant stirring with thrombin, AYPGKF, U46619, ADP (in the presence of fibrinogen), or convulxin, at the concentrations indicated in the figure, in a Chronolog Aggregometer (Mascia Brunelli). Platelet aggregation was monitored continuously over 5 minutes.

Measurement of TxA2 generation.

Platelets (0.05 ml, 4×10^6 platelets/ml) were stimulated with increasing concentrations of thrombin for 15 minutes at 37°C. Reaction was stopped by the addition of 2.5 mM EDTA and 1 mM aspirin. Cells were removed by centrifugation, and supernatants collected and used for the determination of the stable non enzymatic hydratation product of TxA2, TxB2, with a commercial enzyme immunoassay kit, according to the manufacturer’s instructions.
Flow cytometry.

Samples of washed platelets (10^6 cells in 0.05 ml of HEPES buffer containing 1 mM CaCl_2, 1 mM MgCl_2 and 0.2% BSA), untreated or activated with different doses of thrombin, AYPGKF, or convulxin, were labeled for 10 minutes at room temperature with different specific antibodies: PE-conjugated JON/A, FITC-conjugated anti-P-selectin, or FITC-conjugated fibrinogen. Samples were immediately analyzed by flow cytometry using FACSCalibur instrument equipped with CellQuest Pro software (BD Bioscience, Milan Italy). Data analyses were performed using the FlowJo 7.6.1 software (Tree Star).


Agonist-induced release of [14C]serotonin from metabolically labeled platelets was performed as previously described.25

Platelet pulmonary thromboembolism.

Collagen plus epinephrine-induced pulmonary thromboembolism was carried out in Pyk2 KO and in wild type mice essentially as previously described.26,27 Briefly, mice were challenged with 0.1 ml of a mixture containing 200µg/ml collagen and 1.2 µg/ml epinephrine, rapidly injected into one of the tail veins. The mortality of mice in each group was monitored over 15 minutes and data are presented as percentage of animals dead over total number of animals tested. At the end of each experimental session surviving animals were sacrificed by an overdose of anesthesia.

Lung histology.

Two minutes after the thrombotic challenge, mice were rapidly killed by an overdose of anesthesia and the chest was opened, trachea was cannulated and lungs were perfused with a fixing solution (10% formalin buffered with calcium carbonate). The trachea was then ligated and removed together with the lungs which were rinsed in cold saline and then fixed in 10% formalin for at least 24 hours. The right-lower lobe was embedded in paraffin and several sections, 5-6 µm thick, were cut and stained with hematoxylin and eosin to reveal platelet thrombi. The specimens were examined under a light microscope (Wild-Leitz, Heerbrugg, West Germany) by a pathologist unaware of the experimental groups. At least ten fields, at a magnification of 400x, were observed for every specimen. The total number of identifiable lung vessels per field was counted and the percentage of them occluded by platelet thrombi was annotated.26,27
Femoral artery thrombosis.

Photochemical-induced femoral artery thrombosis was induced in anesthetized mice by a method previously described. Briefly, mice were anesthetized by xylazine (5 mg/kg ip) and Ketamine (60 mg/kg ip) and placed on a heated operating table. A 25G needle venous butterfly was inserted in one of the tail veins for the infusion of rose Bengal. The left femoral artery was carefully exposed, and a laser Doppler probe (Transonic System Inc, Ithaca, NY, USA) was positioned onto the branch point of the deep femoral artery, distal to the inguinal ligament, for monitoring blood flow. The exposed artery was irradiated with green light (wavelength 540 nm) of a Xenon lamp (L4887, Hamamatsu Photonics, Hamamatsu, Japan) equipped with a heat-absorbing filter via a 3 mm diameter optic fibre attached to a manipulator. Light irradiation was protracted for 20 minutes; the infusion of rose Bengal (20 mg/kg) was started 5 min after the beginning of irradiation and lasted for 5 minutes. The end point was set as the cessation of blood flow for >30 seconds; in case no occlusion occurred after 30 minutes, the time was recorded as 30 minutes.

Tail bleeding time.

Mice were positioned in a special immobilization cage which keeps the tail of the animal steady and immersed in saline thermostated at 37°C. After two minutes the tip of the tail was transected with a razor blade at 2 mm from its end. The tail was immediately reimmersed in thermostated saline and the time taken to stop bleeding was measured; the end point was the arrest of bleeding lasting for more than 30 seconds. Bleeding was recorded for a maximum of 900 seconds.

Electron microscopy.

Resting gel filtered platelets were fixed for 4 hrs at 4°C, using cacodylate 0.1N-HCl buffer (pH 7.4) containing 4% wt/vol of glutaraldehyde. The samples were then washed, maintained in cacodylate buffer for further 4 hrs, then placed in 1% osmium tetroxide and pelleted by centrifugation at 10,000xg for 30 seconds. Ultrathin sections of the platelet pellets were stained with uranyl acetate and lead citrate and observed with a Phillips Optic EM208 transmission electron microscope at 80kv. For the measurement of platelet size and granules content, micrographs of fifty platelets from wild type and Pyk2 knockout mice were analyzed from 5 different slides for each strain. Data are expressed as means±SEM.
Data and statistical analysis.

All the reported figures are representative of at least three different experiments. Statistical analysis was performed using Prism 4 GraphPad software, and the data were compared by unpaired t-test.
RESULTS

Mice lacking Pyk2 are protected from thrombosis.

Pyk2 knockout mice were viable and fertile, and did not manifest any evident bleeding tendency or thrombotic events over their lifespan. No significant differences in the number of circulating platelets and white cells were observed between wild type and Pyk2 knockout mice (Fig. 1A). Immunoblotting analysis revealed that the absence of Pyk2 in platelets was not compensated by an altered expression of the related focal adhesion kinase FAK (Fig. 1B). No significant differences in the surface expression of some major platelet glycoproteins, including CD9, GPVI, CD42b (GPIbα), CD41 (αIIb subunit), CD49b (α2 subunit), and GPV were observed between wild type and Pyk2 knockout mice (Fig. 1C). Platelet size and morphology were comparable in wild type and Pyk2 knockout mice, as revealed by flow cytometry (data not shown), as well as by electron microscopy (Fig. 1Di). The average diameter of platelets from Pyk2-deficient mice was very similar to that platelets from wild type mice (wild type: 2.55 ± 0.35 µm; Pyk2 knockout: 2.61 ± 0.47 µm, Fig. 1Dii), and the number of α- and dense-granules was comparable in both genotypes (Fig 1Diii). The tail bleeding time was slightly prolonged in Pyk2 knockout mice, but only 4 mice out of the 14 examined had a bleeding time longer than 15 minutes (Fig. 1E), indicating that Pyk2 plays a significant, but not essential role in primary haemostasis.

To investigate the possible involvement of Pyk2 in thrombus formation in vivo, we analyzed a model of photochemical induced femoral artery thrombosis in mice infused with rose Bengal. The formation of an occlusive thrombus was monitored by measuring the blood flow on the exposed artery with a laser Doppler probe. In wild type mice, blood flow stopped within 15 minutes (14.00 ± 1.58 minutes, n= 5) (Fig. 2A). By contrast, in Pyk2 knockout mice the time required for artery occlusion was significantly prolonged up to about 30 minutes (28.20 ± 1.79, n=5, p<0.001), indicating that Pyk2 plays an important role in arterial thrombosis (Fig. 2A).

The reduced thrombotic phenotype of Pyk2 knockout mice was confirmed in pulmonary thromboembolism model. Upon injection of a mixture of collagen plus epinephrine, mortality of Pyk2 knockout mice was lower compared to wild type littermates (Fig. 2B). Importantly, histological analysis of slices of isolated lungs collected 2 minutes after the injection of collagen plus epinephrine showed that the percentage of vessels occluded by platelet thrombi was significantly lower in Pyk2 knockout than in wild type mice (Fig. 2C
and 2D). Altogether these results indicate that Pyk2 plays a relevant role in thrombus formation in vivo.

**Impaired platelet activation and aggregation in the absence of Pyk2.**

To characterize the molecular mechanism for the implication of Pyk2 in thrombosis, we analyzed aggregation of washed platelets from Pyk2 knockout mice. Figure 3 shows that aggregation induced by low doses of thrombin was strongly impaired in Pyk2-deficient platelets, but this effect was overcome by increasing the concentration of the agonist. Accordingly, aggregation in response to the weaker, but selective PAR4 stimulating peptide AYPGFK was almost completely abolished even when relatively high doses of agonist were used. A defective platelet aggregation in the absence of Pyk2 was also observed upon stimulation with low doses of the TxA2 analogue U46619 and, to a lesser extent, ADP (Fig. 3). By contrast, aggregation triggered by convulxin was not significantly different between wild type and Pyk2-deficient platelets (Fig. 3), and it was normal also upon stimulation with collagen or collagen-related peptide (data not shown). These results indicate that Pyk2 may not be essential for GPVI-induced platelet aggregation, but certainly plays a relevant role downstream of GPCRs, including the thrombin receptor PAR4. For this reason, our subsequent investigations were mainly focused on thrombin-stimulated platelets.

We first analyzed whether the reduced aggregation observed in the absence of Pyk2 was associated to an impaired activation of integrin αIIbβ3, by measuring binding of the conformational-dependent antibody JON/A. Figure 4A shows that JON/A binding to Pyk2-deficient platelets stimulated with 0.05 or 0.1U/ml thrombin was strongly reduced compared to platelets from wild type mice (by about 90 and 40%, respectively). Lower doses of thrombin were unable, in our experimental conditions to produce detectable JON/A binding (data not shown), while a higher dose of thrombin (0.5U/ml) caused normal integrin αIIbβ3 activation even in the absence of Pyk2 (Fig. 4A). Moreover, platelet stimulation with the GPVI agonist convulxin promoted inside-out activation of integrin αIIbβ3 independently of Pyk2 (Fig. 4A). In addition, JON/A binding induced by ADP or U46619, alone or in combination, was not significantly altered in Pyk2-deficient platelets (data not shown). The role of Pyk2 in integrin αIIbβ3 inside-out activation downstream of PAR4, but not of GPVI, was confirmed by measuring the binding of FITC-fibrinogen to AYPGFK- or convulxin-stimulated platelets (Fig. 4B).
Pyk2 regulates cPLA$_2$ phosphorylation and TxA$_2$ production in thrombin-stimulated platelets

It is known that platelet aggregation by low doses of agonists relies on autocrine stimulation by platelet-released messengers, such as ADP or TxA$_2$. Therefore, we compared granule secretion and TxA$_2$ production in wild type and Pyk2-deficient platelets stimulated with thrombin. Analysis of P-selectin exposure revealed a modest, albeit statistically significant, reduction of platelet $\alpha$-granules secretion in the absence of Pyk2, especially at the lowest dose of the agonist analyzed (Fig. 5A), but was not observed upon stimulation of GPVI (data not shown). Similarly, thrombin-induced release of serotonin from dense granules was only slightly impaired in Pyk2-deficient platelets (Fig. 5B). By contrast, measurement of accumulation of the stable metabolite TxB$_2$ in the supernatant of stimulated platelets revealed that thrombin-induced production of TxA$_2$ was dramatically impaired in the absence of Pyk2 (Fig 5C). These results demonstrate that Pyk2 has a minor role in platelet secretion, but is an important regulator of TxA$_2$ synthesis in thrombin-stimulated platelets.

Synthesis of TxA$_2$ initiates with the release of arachidonic acid by cPLA$_2$, which is activated by intracellular Ca$^{2+}$ as well as by phosphorylation on Ser505.$^{29-30}$ In platelets, cPLA$_2$ phosphorylation is mainly mediated by p38MAPK.$^{31-34}$ Figure 6A shows that thrombin-induced cPLA$_2$ phosphorylation on Ser505 was significantly reduced in Pyk2-deficient platelets compared to wild type littermates. In agreement with previous findings we confirmed that cPLA$_2$ phosphorylation in thrombin-stimulated platelets was mediated by p38MAPK, as it was prevented by the specific inhibitor SB203580 (data not shown). In nucleated cells, a functional link between Pyk2 and different MAP kinases has been very well documented.$^{7,35-39}$ Therefore, we analyzed thrombin-induced phosphorylation of both p38MAPK and ERK1/2. While the kinetics and the extent of ERK1/2 phosphorylation was comparable in wild type and Pyk2-deficient platelets, phosphorylation of p38MAPK was significantly reduced in the absence of Pyk2, at all the time points analyzed (Fig. 6B and Fig. 6C). These results indicate that p38MAPK links Pyk2 to cPLA$_2$ activation in thrombin-stimulated platelets.

The defective p38MAPK-cPLA$_2$ pathway is responsible for the impaired platelet aggregation of Pyk2-deficient platelets.

We hypothesized that the reduced production of TxA$_2$ could explain the impaired aggregation of Pyk2-deficient platelets stimulated with thrombin. In support to this possibility, we found that the extent of thrombin-induced aggregation of wild type platelets
was reduced to a level comparable to that seen in Pyk2-deficient platelets when TxA2 production was blocked by preincubation with aspirin (Fig. 7A). However, Pyk2-deficient platelets aggregated normally upon direct stimulation with arachidonic acid (Fig. 7A). Aggregation of wild type platelets with the p38MAPK inhibitor SB203580 was comparable to that of untreated Pyk2-deficient platelets, but SB203580 caused only a minimal and not significant further reduction of aggregation of platelets lacking Pyk2 (Fig. 7B). Finally, we observed that the defect of thrombin-induced aggregation caused by the absence of Pyk2 could be significantly, albeit not completely, overcome by the addition of a subtreshold dose of the TxA2 mimetic U46619. Figure 7C shows that 25 nM U46619 was unable to trigger aggregation of Pyk2-deficient platelets, but when added together with a low dose of thrombin, it allowed platelets to aggregate to an extent comparable to that induced by thrombin alone on wild type platelets.
DISCUSSION

In this study we have used a knockout model to investigate the role of the focal adhesion kinase Pyk2 in platelet function in vivo and ex vivo. The results show that Pyk2 is an important regulator of platelet aggregation triggered by mild stimulation of GPCRs, including the thrombin receptor PAR4. At the molecular level, our results reveal the implication of Pyk2 in the signaling pathway for thrombin-induced activation of p38MAPK, and phosphorylation of cPLA2, leading to TxA2 production. Moreover, we have demonstrated that Pyk2 is involved in thrombus formation in vivo.

The activation of Pyk2 in platelets upon stimulation by a variety of agonists was reported shortly after the discovery of this kinase, almost 15 years ago. Since then, however, our knowledge on the role of this kinase in platelet function has not advanced significantly, mainly because of the lack of suitable tools and inhibitors. Some peculiar properties of Pyk2, however, suggest that this kinase may represent an important element in many signaling pathways for platelet activation. For instance, Pyk2 is unique in that it can be activated by intracellular Ca2+, and, thus, it may link G-protein-mediated phospholipase C activation and stimulation of protein tyrosine phosphorylation. In this context, we demonstrated herein that Pyk2 is critically involved in platelet activation by thrombin. It was previously shown that thrombin induces a rapid and robust, Ca2+-dependent phosphorylation and activation of Pyk2. In this study, we found that platelets from Pyk2 knockout mice show a defective aggregation in response to low, but not high, concentrations of thrombin and to PAR4-activating peptide. This effect was likely the consequence of an impaired integrin αIIbβ3 inside-out activation, because a reduced binding of fibrinogen as well as of the conformational dependent antibody JON/A was observed in Pyk2-deficient platelets. Although the effect on thrombin-induced aggregation was definitively more pronounced, an impaired aggregation in the absence of Pyk2 was also detected upon stimulation with low concentration of other agonists, including ADP and U46619, allowing us to extend the importance of Pyk2 in platelet response to different GPCRs-stimulating agonists. Notably, we observed that stimulation of GPVI with collagen, convulxin, or CRP caused normal aggregation of Pyk2-deficient platelets, suggesting that this kinase does not play a central role in ITAM-based signaling in platelets. This conclusion is also supported by the observation that inside-out activation of integrin αIIbβ3 in Pyk2-deficient platelets was reduced upon stimulation with thrombin, but occurred normally in response to convulxin.
We found that Pyk2 deficiency impaired thrombin-induced secretion of both α and dense granules, but this effect, albeit statistically significant, was rather modest, and therefore it is unlikely to contribute to the defective aggregation. By contrast, our results indicate that Pyk2 is a major regulator of TxA2 production in thrombin-stimulated platelets. We propose that the strongly reduced production of TxA2 in Pyk2-deficient platelets is responsible for the defective platelet aggregation in response to thrombin. This conclusion is supported by the evidence that pharmacologic inhibition of TxA2 production in wild type platelet reduces the extent of aggregation induced by thrombin to a level comparable to that seen in the absence of Pyk2. Moreover, the defective thrombin-induced aggregation of Pyk2 deficient platelets can be almost completely rescued by the addition of subtreshold amounts of the TxA2 analogue U46619.

The production of TxA2 is initiated by the action of cPLA2, which releases arachidonic acid from membrane phospholipids. In this study, we report evidence indicating that cPLA2 is actually regulated through Pyk2 in thrombin-stimulated platelets. cPLA2 is typically activated by an increase of intracellular Ca2+ and by phosphorylation on Ser505, which is mediated by different MAP kinases.29,30 In platelets, phosphorylation of cPLA2 has been documented to occur in response to a number of different agonists including thrombin, and to be mainly mediated by p38MAPK, rather than ERK1/2.31-34 We confirmed that thrombin-induced phosphorylation of cPLA2 in mouse platelets is prevented by the specific p38MAPK inhibitor SB203580 (data not shown), and in the present study, we also documented that cPLA2 phosphorylation is significantly reduced in platelets lacking Pyk2. Importantly, we also found that in thrombin-stimulated platelets phosphorylation of p38MAPK, but not ERK1/2, was reduced in the absence of Pyk2. In this context, our results are in line with many previous studies in different nucleated cells, that have consolidated the role of Pyk2 as a major regulator of several MAPK, including p38MAPK.7,35-39 Moreover, we found that the p38MAPK inhibitor SB203580 inhibited thrombin induced aggregation of wild type, but not of Pyk2-deficient platelets. A previous study by Kuliopulos et al. reported that a novel, isoform-specific inhibitor of p38MAKP-α, VX-702, does not prevent platelet aggregation.40 Moreover, considering that VX-702 did not affect collagen-induced aggregation, which is sensitive to aspirin, the role of p38MAPK in TxA2 generation was questioned.40 We confirmed that, differently from SB203580, VX-702 did not inhibit thrombin-induced aggregation (data not shown). Although SB203580 has been clearly shown to inhibit p38MAPK in several studies, this observation opens the possibility that, in addition to the p38MAPK-cPLA2 pathway, Pyk2 deficiency may impair platelet aggregation by targeting
additional steps in TxA2 production and action. The observation that in Pyk2-deficient platelets, cPLA2 phosphorylation and TxA2 generation stimulated by thrombin are almost completely prevented, while p38MAPK activation is reduced only by about 50% seems in line with this possibility. Therefore, although our findings clearly delineate a novel signaling pathways linking Pyk2 to cPLA2 activation in thrombin-stimulated platelets through the regulation of p38MAPK, additional targets for Pyk2 alone in this pathway can also be involved, and remain to be identified.

The reduced ex vivo aggregation of platelets from Pyk2 knockout mice in response to low doses of thrombin, as well as to other GPCR agonists, suggests that Pyk2, which is currently considered as a promising target in cancer,41 may also represent a novel molecular target for anti-thrombotic agents. This possibility is strengthened by the findings of the present study, demonstrating an important role for Pyk2 in thrombosis in vivo. Using a model of laser-induced arterial thrombosis in Pyk2 knockout mice we have shown a significant contribution of this kinase in vessel occlusion. Moreover, we also found that Pyk2 knockout mice are significantly protected against thromboembolism upon injection of a mixture of collagen plus epinephrine, and a significant lower number of occlusive platelet thrombi in the lungs were observed. Collagen plus epinephrine-induced platelet pulmonary thromboembolism model, represents a system in which in vivo platelet activation, in addition to vasoconstriction and/or endothelial damage, plays a central role. This model is sensitive to antiplatelet drugs, and in particular to agents that suppress the synthesis or action of TxA2.42-44 The observation that Pyk2 knockout mice, in which platelet TxA2 synthesis is impaired, are partially protected from the lethal effects of the injection of collagen plus epinephrine is actually consistent with these previous pharmacologic studies. Moreover, the reduction of the number of histologically-detected platelet-rich thromboemboli in lung vessels of Pyk2-deficient mice points towards a primary action of Pyk2 in regulating in vivo platelet function.

Similarly the chemical method adopted in this study to trigger arterial thrombosis in mice and based on the intravenous injection of the photoreactive substance Rose Bengal represents an experimental system known to be sensitive to the pharmacologic inhibition of TxA2.45,46 This model thus appears especially suitable for testing the function of Pyk2, that modulates GPCRs-stimulated platelet responses and TxA2 production. Therefore, altogether, our results are compatible with a model in which Pyk2 contributes to the activation of platelets with a mechanism that could become crucial in vivo in transforming a normal haemostatic response to a mild vessel wall damage into a thrombotic response.47 In a recent study, we have also found that ex vivo thrombus formation under flow on a collagen matrix was strongly reduced.
in the absence of Pyk2.\textsuperscript{14} Our findings also indicate that Pyk2 is relevant, but not essential for haemostasis, as the bleeding time was mildly increased in Pyk2 knockout mice. However we observed a high variability in the time required to stop bleeding in the absence of Pyk2, and actually a clear bleeding tendency was observed only in about 1/3 of the analyzed mice.

In conclusion, our study has documented that the Ca\textsuperscript{2+}-dependent focal adhesion kinase Pyk2 plays an important role in platelet activation induced by thrombin and is implicated in thrombus formation \textit{in vivo}. These observations indicate that Pyk2 may represent a novel target to modulate GPCRs-stimulated platelet responses. Moreover, the regulation of mechanisms that contribute to amplify the platelet response during activation but do not significantly contribute to the primary response to strong agonists may represent a way to obtain an antithrombotic effect without significantly impairing haemostasis.
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Contribution of Authors
Ilaria Canobbio: designed and performed experiments, analyzed data
Lina Cipolla: designed and performed experiments, analyzed data
Alessandra Consonni: designed and performed experiments, analyzed data
Stefania Momi: designed and performed experiments, analyzed data, wrote the manuscript
Gianni Guidetti: performed experiments, analyzed data
Barbara Oliviero: performed experiments, analyzed data
Marco Falasca: contributed vital new reagents, analyzed and interpreted the data, helped draft the output
Mitsuhiko Okigaki: contributed vital new reagents, edited the manuscript
Cesare Balduini: analyzed data, edited the manuscript
Paolo Gresele: analyzed data, edited the manuscript
Mauro Torti: designed research, analysed data, wrote the manuscript, overall direction

Conflict-of-interest disclosure
The authors declare no competing financial interests.
REFERENCES


FIGURES LEGENDS

Figure 1. Characterization of platelets from Pyk2 knockout mice.
A. Platelets and white cells count in whole blood from wild type (black bars) and Pyk2 knockout (white bars) mice. Results are the mean ± SD of determination performed in 10 different mice.
B. Analysis of Pyk2, FAK and tubulin expression in platelets from wild type (WT) and Pyk2 knockout (Pyk2 KO) mice, by immunoblotting with specific antibodies as indicated on the right.
C. Surface expression of different glycoproteins on wild type (black bars) and Pyk2 knockout (white bars) mice, determined by flow cytometry analysis with specific antibodies. Data are expressed as mean fluorescence intensity ± SD of three different experiments performed in duplicate.
D. Electron microscopy analysis of wild type (WT) and Pyk2-deficient (Pyk2 KO) platelets. Representative images at different magnitudes (7,000x and 22,000x respectively) are reported in (i). Measurement of the mean platelet diameter is reported in (ii), and quantification of α- and dense-granules is shown in (iii). Data have been obtained from the analysis of fifty platelets from 5 different slides for each genotype, and are expressed as means ± SEM.
E. Tail bleeding time determined in groups of 13 wild type (WT) and 14 Pyk2 knockout mice (Pyk2 KO). Each symbol represents 1 animal. Statistic analysis revealed a significant difference between the two groups of animals (p<0.01).

Figure 2. Analysis of thrombus formation in Pyk2 knockout mice.
A. Photochemical-induced arterial thrombosis assesses as time required for occlusion of femoral artery measured by laser Doppler in wild type (black bars) and Pyk2 knockout (white bars) mice. Data are the mean ± SD of measurements performed on 5 animals for both genotypes. ***: p<0.001
B. Pulmonary thromboembolism-associated mortality caused by injection of epinephrine plus collagen in wild type (black bars, 10 animals analyzed), and Pyk2 knockout (white bars, 11 animals analyzed) mice.
C. Percentage of vessels occluded by platelet thrombi in the lungs of wild type (black bars) and Pyk2 knockout (white bars) mice upon injection of collagen and epinephrine, as determined by count in 10 microscopic fields for each lung section. Data are reported as the mean ± SD ( * p< 0.05)
D. Representative histology images (100x) of lungs from wild type and Pyk2 knockout mice after collagen plus epinephrine injection. Staining is with hematoxylin and eosin, and arrows indicate the platelet rich thrombi.

Figure 3. Aggregation of Pyk2-deficient platelets.
Washed platelets from wild type (WT) and Pyk2 knockout (Pyk2 KO) mice were stimulated in an aggregometer with different concentrations of thrombin, AYPGFK, ADP, U46619, or convulxin, as indicated in each panel. For ADP-induced aggregation 200 μg/ml fibrinogen was added to the platelet suspension before stimulation with the agonist. Aggregation was monitored as increase of light transmission up to 5 minutes. Tracings in the figure are representative of at least 3 different experiments.

Figure 4. Analysis of integrin αIIbβ3 inside-out activation.
Flow cytometry analysis of PE-JON/A binding (panel A) or FITC-labeled fibrinogen binding (panel B) to wild type (black bars) and Pyk2 knockout (white bars) platelets stimulated with the indicated doses of thrombin, AYPGKF, or convulxin (CVX). Data are expressed as mean ± SD of three-to eight different experiments. ***: p< 0.001; **: p< 0.01

Figure 5. Analysis of platelet secretion and TxA2 production.
A. Flow cytometry analysis of P-selectin exposure in platelets stimulated with the indicated doses of thrombin. Data are expressed as mean fluoresce intensity, and are the mean ± SD of 4 experiments. *: p< 0.05; **: p< 0.01; ***: p< 0.005. Black bars: wild type platelets; white bars: Pyk2-deficient platelets.
B. Analysis of thrombin-induced release of [14C]serotonin from wild type (black bars) and Pyk2 knockout (white bars) platelets. The release of serotonin in the supernatant of platelets stimulated with the indicated doses of thrombin for 1 minute is expressed as percentage of the total incorporated radioactivity upon subtraction of the values measured in the supernatant of resting platelets. Data are expressed as mean ± SD of three different experiments. *: p< 0.05; ***: p< 0.005.
C. Accumulation of TxB2 in the supernatant of wild type (black bars) and Pyk2 knockout (white bars) platelets, stimulated with the indicated doses of thrombin for 15 minutes. Data are expressed as mean ± SD of 3 different experiments. *: p<0.05; **: p<0.01
Figure 6. Analysis of cPLA2, ERK, and p38MAPK phosphorylation.
A. Washed platelets from wild type (WT) and Pyk2 knockout (Pyk2 KO) mice were stimulated with 0.05 U/ml thrombin for 0, 5, or 15 minutes, as indicated. The phosphorylation of cPLA2 was evaluated by immunoblotting with a phosphospecific antibody, followed by a subsequent immunoblotting with anti-cPLA2, as indicated on the right. Quantification of cPLA2 phosphorylation was performed by densitometric analysis of the immunoblots, and data are reported in the histogram as mean ± SD of 4 different experiments. ***: p<0.001.
B and C. Phosphorylation of ERK (panel B) and p38MAPK (panel C) in platelets from wild type (WT) and Pyk2 knockout mice (Pyk2 KO) stimulated with 0.05 U/ml thrombin for 0.5, 1, and 2 minutes was analyzed by immunoblotting with phosphospecific antibodies as indicated on the right. Blot with anti-p38MAPK or anti-ERK were performed as control for equal loading. Quantitative evaluation of protein phosphorylation is reported in the histograms as the means ± SD of 3 different experiments. *: p<0.05, **: p<0.01

Figure 7. The reduced TxA2 production is responsible for the defective aggregation of Pyk2-deficient platelets.
Aggregation of platelets from wild type (WT) and Pyk2 knockout (Pyk2 KO) mice was monitored as increase of light transmission up to 5 minutes, upon stimulation with thrombin, arachidonic acid, or U46619, as indicated in each panel. When indicated, platelets were preincubated with aspirin (ASA, 1 mM, 30 minutes) or SB203580 (10 μM, 10 minutes). Equivalent volumes of DMSO as vehicle were added to control samples. The arrows indicate the addition of the agonists. Traces in the figures are representative of at least 3 different experiments.
figure 1

A

B

C

D

(i)

WT

Pyk2 KO

(ii)

WT

Pyk2 KO

(iii)

WT

Pyk2 KO
Figure 2

A

B

C

D

WT

Pyk2 KO

WT

Pyk2 KO

WT

Pyk2 KO
figure 3

thrombin, U/ml

0.05 0.035 0.02 0.0125

AYPGFK, mM

1 0.5 0.25

ADP, μM

10 5 0.5

U46619, μM

0.5 0.25 0.1

convulxin, ng/ml

50 25 10

5 minutes
Figure 4

A

![Graph A showing active αIIbβ3 MFI](image)

B

![Graph B showing fibrinogen FcγR binding, MFI](image)
Figure 5

A

Protease exposure, MFI over basal

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B

$^{14}$C-serotonin secretion, %

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C

$\text{TXB}_2$ (pg/ml)

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* denotes significance.
figure 6

A

B

C
figure 7

A

B

C

light transmission, %

5 minutes

WT

Pyk2 KO

WT + ASA

100

+ thrombin 0.025 U/ml

5 minutes

+ thrombin 0.025 U/ml

5 minutes

+ thrombin 0.01 U/ml

5 minutes

+ thrombin 0.01 U/ml

5 minutes

+ thrombin 0.01 U/ml

5 minutes

+ thrombin 0.01 U/ml

5 minutes
Impaired thrombin-induced platelet activation and thrombus formation in mice lacking the Ca$^{2+}$-dependent tyrosine kinase Pyk2

Ilaria Canobbio, Lina Cipolla, Alessandra Consonni, Stefania Momi, Gianni Guidetti, Barbara Oliviero, Marco Falasca, Mitsuhiko Okigaki, Cesare Balduini, Paolo Gresele and Mauro Torti