The AMP-activated protein kinase (AMPK) \(\alpha2\) subunit is involved in platelet signaling, clot retraction and thrombus stability

Voahanginirina Randriamboavonjy,\(^1\) Johann Isaak,\(^1\) Timo Frömel\(^1\), Benoit Viollet,\(^2,3\) Beate Fisslthaler,\(^1\) Klaus T. Preissner,\(^4\) Ingrid Fleming\(^1\)

\(^1\)Institute for Vascular Signalling, Centre for Molecular Medicine, Johann Wolfgang Goethe University, Frankfurt am Main, D-60596, Germany and \(^2\)Institut Cochin, Université Paris Descartes, CNRS (UMR 8104), and \(^3\)Inserm, U567, Paris, France and \(^4\)Institute for Biochemistry, Medical Faculty, Justus-Liebig-University, Giessen, D-35392, Germany.

**Short title:** AMPK\(\alpha2\) in platelet signaling and function

Address correspondence to: Ingrid Fleming PhD, Institute for Vascular Signalling, Centre for Molecular Medicine, Johann Wolfgang Goethe University, Theodor-Stern-Kai 7, D-60596 Frankfurt am Main, Germany. Tel: (+49) 69 6301 6972; Fax: (+49) 69 6301 7668; Email: fleming@em.uni-frankfurt.de.
Abstract

The AMP-activated protein kinase (AMPK) is a regulator of energy balance at both the cellular and whole-body levels but little is known about the role of AMPK in platelet activation. Here we report that both the AMPKα1 and α2 isoforms are expressed by human and murine platelets and that thrombin elicits the phosphorylation of AMPKα as well as the upstream kinase; liver kinase B1 (LKB1). In human platelets, the kinase inhibitors iodotubercidin and compound C significantly inhibited thrombin-induced platelet aggregation and clot retraction without affecting the initial increase in [Ca^{2+}]. Clot retraction was also impaired in platelets from AMPKα2−/− mice but not from wild-type littermates or AMPKα1−/− mice, moreover, rebleeding was more frequent in AMPKα2−/− mice and the FeCl₃-induced thrombi formed in AMPKα2−/− mice were unstable. Mechanistically, AMPKα2 was found to phosphorylate (in vitro) the Src-family kinase, Fyn, and AMPKα2 deletion resulted in the attenuated threonine phosphorylation of Fyn, as well as the subsequent tyrosine phosphorylation of its substrate, β3 integrin. Taken together, our data indicate that the AMPKα2, by affecting the phosphorylation and activity of Fyn, plays a key role in platelet αIIbβ3 integrin signaling leading to clot retraction and thrombus stability.

Key words: AMPK, clot retraction, β₃ integrin, Fyn
Introduction

The AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine protein kinase consisting of the catalytic subunit (α) and two regulatory subunits (β and γ) that exist as multiple isoforms and splice variants. Each subunit within the heterotrimeric AMPK complex has a distinct structure and function and their interaction is necessary for the modulation of kinase activity. As its name suggests, the AMPK is activated in many different cell types by increased intracellular concentrations of AMP, and is generally referred to as a “metabolite-sensing kinase”. Indeed, the AMPK is activated following heat shock, vigorous exercise, hypoxia/ischemia and starvation, and appears to be a metabolic master switch, phosphorylating key target proteins that control flux through different metabolic pathways (for recent detailed review see Steinberg and Kemp1).

Given that activity is clearly associated with the phosphorylation of the α subunit it was initially assumed that an AMPK kinase (AMPKK) rather than the AMPK itself is activated by AMP. It now seems that AMP binds to the γ subunit, allosterically activating it, so that the α subunit can be phosphorylated at the same time as indirectly inhibiting dephosphorylation.2

There are several kinases which phosphorylate the AMPKα subunits including the constitutively active tumor suppressor gene product; liver kinase B1 (LKB1), and the Ca²⁺/calmodulin-dependent protein kinase kinase β (CaMKKβ). The activation of the latter following cell stimulation with Ca²⁺ ionophores or Ca²⁺ elevating agonists, results in the phosphorylation and activation of the AMPK without detectable changes in the AMP/ATP ratio.3 There are two different AMPKα isoforms that are differentially expressed in different tissues; the α1 isoform predominates in adipose tissue, while the AMPKα2, which determines whole body insulin sensitivity,4 is expressed in skeletal muscle and to a lesser extent in cardiomyocytes.5 Whether or not the AMPKαs are preferential substrates for one or more putative AMPKK remains to be clarified.

Platelets play a key role in thrombosis and hemostasis, and although platelet activation is an energy-consuming process,6,7 surprisingly little is known about the consequences of platelet
activation on the AMPK or the role of the AMPK in the regulation of platelet function. In fact, only one study reports the presence of the kinase in washed human platelets and its activation by insulin. Therefore, the aim of the present study was to determine the role(s) of the AMPKα1 and AMPKα2 in regulating human and murine platelet function.

Materials and Methods

Reagents

The anti-AMPKα1 antibody was generated by Eurogentec (Cologne, Germany), the antibodies against AMPK-α2 and phospho-Thr12-Fyn were from Santa Cruz Biotechnology (Heidelberg, Germany), the antibody recognizing total Fyn antibody was from Abcam (Cambridge, UK) and the antibodies recognizing phospho-Thr172 AMPK, phospho-Ser79 ACC and phospho-Thr189 LKB1 were from Cell Signaling (Frankfurt am Main, Germany). The phospho-Tyr747 β3-integrin antibody was from BioSource (Karlsruhe, Germany) and the β3-integrin antibody from Epitomics (Biomol, Hamburg, Germany). The antibodies against acetyl-CoA carboxylase (ACC), LKB1 and phospho-threonine Fyn were from Upstate Biotechnology (Biomol, Hamburg, Germany). Recombinant AMPKα1, AMPKα2 and STO-609 were from Calbiochem (Bad Soden, Germany), recombinant Fyn was from Upstate Biotechnology (Biomol, Hamburg, Germany). Thrombin was from Hemochrom Diagnostica GmbH (Essen, Germany), the 3,3'-dihexyloxacarbocyanine iodide (DIOC6), was from Molecular Probes (Karlsruhe, Germany). All other reagents were from Sigma (Steinheim, Germany).

Animals

AMPKα1−/− (mixed C57BL6 and SV-129 background), and AMPKα2−/− mice (C57BL6 background) were generated as described. Animals and their respective wild-type littermates (AMPKα1+/+ and AMPKα2+/+) were housed in conditions that conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication no. 85-23). Both the university animal care committee and the federal...
authority for animal research (Regierungspräsidium Darmstadt, Hessen, Germany) approved the study protocols.

**Bleeding and re-bleeding time**

Mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg) and placed on a heated mat. A one millimeter section of the tail tip was cut and the tail tip immediately immersed in sterile saline at 37°C. The bleeding time i.e. the time between the flows of blood to the cessation of bleeding was recorded. The mice were monitored for an additional 10 minutes and if tail bleeding restarted, incidence was recorded (re-bleeding).

**Platelet isolation**

*Human platelets:* Platelets were obtained by centrifugation (900g, 7 min) of platelet-rich plasma, as described. The resulting pellet was washed in Ca2+-free HEPES buffer (mmol/L: NaCl, 136; KCl, 2.6; MgCl2, 0.93; NaH2PO4, 3.26; glucose, 5.5; HEPES, 3.7; pH 7.4 at 37°C) and samples were either lysed for Western Blot analysis, or resuspended in HEPES buffer to a density of 4x10^8 platelets/mL for the measurement of intracellular Ca2+ or platelet aggregation as described.8;10

*Murine platelets:* Mice were anesthetized with isoflurane and blood was collected via cardiac puncture into a syringe containing 10% acidic citrate dextrose (120 mmol/L sodium citrate, 110 mmol/L glucose, 80 mmol/L citric acid) as anticoagulant. Blood was pooled from 3-4 mice from each genotype and platelets were prepared from whole blood by differential centrifugation and resuspended in Ca2+-free HEPES buffer to a density of 8x10^8 platelets/mL.

**Clot retraction**

Platelet-rich plasma (adjusted to 3x10^8 platelets/mL for human platelets and 5x10^8 platelets/mL for murine platelets, 300 µL) obtained by centrifugation of whole blood at 250g for 10 minutes, was stimulated with thrombin (0.3 U/mL for human and 1U/mL for murine) in the presence of CaCl2 (2 mmol/L) and 2 µL erythrocytes to enhance the contrast of the clot. The clots were allowed to retract for up to 3 hours at 37°C and were photographed at
different time points. The extent of retraction was quantified using TINA20 software (Raytest GmbH, Straubenhardt, Germany).

Immunoblotting

Washed human or murine platelets were solubilized in Triton X-100 lysis buffer, 50 µg soluble protein (approximately 5x10^7 platelets) were separated by SDS-PAGE and subjected to Western blotting as described. Proteins were visualized by enhanced chemiluminescence using a commercially available kit (Amersham, Freiburg, Germany).

Thrombus formation in a FeCl₃-induced carotid artery model

Thrombus formation in vivo was assessed as described. Briefly, mice were anesthetized by intraperitoneal injection of ketamine and xylazine and placed on a heated mat. The fluorescent dye DIOC₆ (5 µL of a 100 µmol/L solution/g of body weight) was injected into the jugular vein to allow visualization of the thrombus. Thereafter, a segment of the right carotid artery was exposed and injury was induced by the topical application of FeCl₃ for 2 minutes (Whatmann paper 1 mm² soaked with 0.2 µL of 10% FeCl₃). The artery was then rinsed with saline and thrombus formation was monitored for 30 minutes by placing the carotid artery under a fluorescence microscope equipped with a camera (AxioScope, Carl Zeiss, Jena, Germany). Fluorescent images were acquired sequentially (1 image/second) and thrombus size was quantified using AxioVision 4.7 imaging software (Carl Zeiss).

In vitro kinase assay.

AMPK activity was initiated by incubating recombinant AMPKα1 or AMPKα2 protein (100 ng) with recombinant Fyn (100 ng) in 30 µl of assay buffer (in mmol/L HEPES, 40; pH 7; NaCl, 80; MgCl₂, 5; DTT, 2; EDTA, 0.8; and 8% glycerin, 200 µmol/L, 5’AMP and 1µCi ^32P-γATP) for 40 minutes at 37°C. The reaction was terminated with SDS sample buffer and samples were separated by SDS-PAGE. The gel was dried before being exposed to an X-ray film. Parallel experiments were performed in the presence of unlabeled ATP and Fyn phosphorylation determined by Western blotting.
Statistical analysis

Data are expressed as mean ± SEM, and statistical evaluation was performed using Student’s t test for unpaired data, one-way analysis of variance (ANOVA) followed by a Bonferroni t test, where appropriate. Values of P<0.05 were considered statistically significant.

Results

Thrombin induces AMPKα phosphorylation via the activation of LKB1

Washed human platelets expressed both the α1 and α2 subunits of the AMPK (Figure 1A). A low level of AMPK phosphorylation was detectable in unstimulated platelets but the addition of thrombin (0.03 to 0.3 U/mL) concentration-dependently increased the phosphorylation of the AMPK on Thr172. Phosphorylation is reported to be essential for AMPK activation, and was paralleled by an increase in kinase activity as evidenced by the phosphorylation of the AMPK substrate, ACC (Figure 1B).

Several kinases are able to phosphorylate AMPKα subunits, including LKB1 and CaMKKβ. Although the thrombin-induced activation of the AMPK in endothelial cells is dependent on the activation of CaMKKβ, the CaMKK inhibitor STO-609 did not inhibit, but in fact tended to potentiate, the thrombin-induced activation of the AMPK as well as the thrombin-induced aggregation of human platelets (Supplementary Figure I). However, stimulation of washed human platelets with thrombin markedly increased the phosphorylation of LKB1 indicating that LKB1 is most probably the AMPKK under these conditions (Figure 2A). Since the phosphatidylinositol 3-kinase (PI3-K) is known to be activated by thrombin, and has been reported to be upstream of the AMPK in insulin-stimulated platelets, we determined whether or not the PI3-K affects the LKB1-AMPK pathway. Inhibition of the PI3-K using a low concentration of wortmannin (20 nmol/L) prevented the thrombin-induced phosphorylation of LKB1, as well as that of the AMPK and ACC in human platelets (Figure 2B).
Role of AMPK in thrombin-induced aggregation and clot retraction

We next assessed the consequences of AMPK inhibition on thrombin-induced platelet aggregation and Ca^{2+}-signaling. Stimulation of washed human platelets with thrombin (0.01-0.3 U/mL) led to a concentration-dependent aggregation (Figure 3A). Pre-incubation of platelets with either of the AMPK inhibitors; iodotubercidin (1 µmol/L) or compound C (10 µmol/L), significantly attenuated the aggregation induced by thrombin concentrations up to 0.3 U/mL (Figure 3A). The aggregation induced by the highest concentration of thrombin (1 U/ml) was, however, not affected by kinase inhibition. Neither iodotubercidin nor compound C affected the thrombin-induced increase in platelet [Ca^{2+}] (Figure 3B) suggesting that the involvement of the AMPK in thrombin-induced aggregation is downstream of the increase in [Ca^{2+}].

Stimulation of human platelet-rich plasma with thrombin (0.3 U/mL) resulted in the formation of platelet clots and their time-dependent retraction. While iodotubercidin and compound C did not affect clot formation, both compounds prevented clot retraction over 60 minutes (Figure 3C).

AMPKα2 not AMPKα1 regulates clot retraction and thrombus stability

As the pharmacological tools that target the AMPK are not specific and there is no possibility to selectively inhibit/downregulate the different α subunits in platelets, we assessed the expression of the AMPK in platelets from wild-type and AMPKα^{-/-} mice. As with washed human platelets, platelets from wild-type mice expressed both AMPKα isoforms (Figure 4A). As expected, no AMPKα1 could be detected in platelets from AMPKα1^{-/-} mice and no AMPKα2 could be detected in platelets from AMPKα2^{-/-} mice. Consistent with the data obtained using human platelets, thrombin-induced aggregation was attenuated in platelet from AMPKα2^{-/-} versus AMPKα2^{+/+} mice (Supplementary Figure 2). Moreover, AMPKα2 activation during aggregation was not restricted to the signaling cascade activated by thrombin as collagen-induced aggregation was also significantly inhibited in platelets from AMPKα2^{-/-} mice (Supplementary Figure 2B). There was no significant difference in the
thrombin-induced aggregation of platelets from AMPKα1+/+ and AMPKα1−/− mice (data not shown). As with Ca2+ signaling in human platelets, which was unaffected by AMPK inhibition, platelet degranulation was also unaffected by AMPKα2 deletion. The thrombin-induced release of ATP (α2+/+: 34.2±1.2 and α2−/−: 35.5±1.8 µmol/µL platelet supernatant, 4 animals) and thromboxane B2 (α2+/+: 15.5±1.1 and α2−/−: 14.0±0.9 ng/mL platelet supernatant, 4 animals) were comparable.

Platelet-rich plasma from AMPKα1−/−, AMPKα2−/− mice and their respective wild-type (AMPKα+/+) littermates was stimulated with thrombin (0.3 U/mL). While maximal retraction was observed in platelet-rich plasma from wild-type and AMPKα1−/− animals (Figure 4B), clot retraction in platelet-rich plasma from AMPKα2−/−-deficient mice failed to occur.

There was no significant difference in the tail bleeding times in the animals studied: average bleeding times were 78±8 and 118±28 seconds in AMPKα2+/− and AMPKα2−/− mice, respectively (n=11). However, re-bleeding was more frequently observed in AMPKα2−/− mice (6 of 10 mice versus 2 of 10 AMPKα2+/− mice) suggesting that AMPK is involved in the stabilization and thrombus consolidation rather than in the formation of the primary platelet plug. Therefore, we assessed thrombus formation and stability in vivo following FeCl3-induced carotid artery injury. While the AMPKα2+/− mice developed a dense thrombus covering the injured surface that attained maximum size approximately 5 minutes after injury, a less compact thrombus with a maximum size recorded after 15 minutes was formed in arteries from AMPKα2−/− mice (Figure 4C). Moreover, large emboli continuously detached from the thrombi formed in AMPKα2−/− mice, indicating clot instability and resulting in a decrease in thrombus size (Figure 4C and Supplementary video 1&2). The thrombus development and stability were similar in AMPKα2+/−, AMPKα1+/− and AMPKα1−/− mice (data not shown).
AMPK and the thrombin-induced phosphorylation of β3 integrin

The phosphorylation of the cytoplasmic domain of β3 integrin on Tyr747 is required for stable platelet aggregation and optimal clot retraction. Therefore, we determined whether or not the altered function of platelets from AMPKα2−/− mice could be linked to changes in integrin phosphorylation.

In washed human platelets, thrombin elicited the concentration-dependent tyrosine phosphorylation of β3 integrin; a response markedly attenuated in the presence of compound C (Figure 5A). Thrombin also stimulated the tyrosine phosphorylation of β3 integrin in platelets from AMPKα2+/+ mice but failed to elicit the same response in platelets from their AMPKα2−/− littermates. The thrombin-induced phosphorylation of β3 integrin was not significantly different in platelets from AMPKα1+/+ and α1−/− mice (Figure 5B). These data indicate that a β3-phosphorylating tyrosine kinase may be a substrate of the AMPKα2 in platelets.

Several Src family kinases (SFK) can be detected in megakaryocytes and platelets and are thought to be involved in regulating platelet function. Therefore, we determined the consequences of SFK inhibition on β3 integrin phosphorylation. Pretreatment of human platelets with PP2 (30 nmol/L), attenuated the thrombin-induced phosphorylation of β3 integrin (Figure 6A) and aggregation; PP2 inhibited the aggregation induced by 0.03 U/mL thrombin by 50.52% (n=4, P<0.05) and that induced by 0.3 U/mL thrombin by 27.31% (n=4, P<0.05). PP2 did not affect the thrombin-induced phosphorylation of either AMPKα1 or AMPKα2 (data not shown). As Fyn can phosphorylate β3 integrin and Fyn is itself regulated by phosphorylation on Thr12, we next determined whether or not a link exists between AMPKα2 and Fyn in platelets.

In an in vitro 32P kinase assay we were able to detect the autophosphorylation of Fyn in the absence of the AMPK but observed a marked increase in phosphorylation in the presence of AMPKα2 (Figure 6B). The latter effect was resistant to the SKF inhibitor PP2 but was
abrogated by iodotubercidin and compound C indicating the AMPK-dependent phosphorylation of Fyn and not the other way around. Experiments repeated in the absence of radioactive ATP but using a phospho-selective threonine antibody confirmed the threonine phosphorylation of Fyn by AMPKα2 (Figure 6B). We were however unable to demonstrate the AMPK-mediated phosphorylation of a purified recombinant Src (data not shown).

We finally assessed the ability of thrombin to elicit the phosphorylation of Fyn on Thr12 in murine platelets and found that, while thrombin induced the phosphorylation of Fyn on Thr12 in platelets from AMPKα2+/+ mice, there was no detectable phosphorylation of this residue in platelets from AMPKα2−/− mice (Figure 6C). No difference in Fyn phosphorylation was detected in AMPKα1+/+ and AMPKα1−/− platelets (data not shown).

Discussion

The results of the present study demonstrate that the α1 and α2 subunits of AMPK are expressed in human and murine platelets. Moreover, platelet activation by thrombin resulted in the phosphorylation of the AMPK, LKB1, AMPK itself and the AMPK substrate, ACC. AMPK activation seems to be required for normal platelet responsiveness as pharmacological inhibition of the kinase attenuated platelet aggregation and clot retraction. Moreover, from studies in AMPKα1+/+ and AMPKα2−/− mice it seems that the α2 subunit plays a key role in the regulation of platelet function and thrombus stabilization by regulating the Fyn-mediated phosphorylation of β3 integrin.

Platelet activation is an energy consuming process,6,7 which makes it logical to assume a potentially important role for the AMPK in the regulation of platelet signaling and function. However, although the AMPK is expressed in platelets, little is known about the predominance of the different α subunits or the mechanisms regulating AMPK activity. We reported previously that platelet stimulation with insulin increased AMPK activity and that AMPK inhibition prevented the anti-aggregatory effects of the hormone8 but more detailed analysis was hampered by the lack of selectivity of the available inhibitors and appropriate mouse models. We now report that both the AMPKα1 and α2 subunits are expressed in...
human and murine platelets and that the kinase can be activated by cell stimulation with thrombin. Indeed, thrombin led to an increase in the phosphorylation of AMPK on Thr172 as well as that of its substrate ACC.

Several AMPK phosphorylating kinases or AMPKKs have been described and on the basis of the literature available we initially speculated that CaMKKβ would be essential for the phosphorylation and activation of the AMPK in platelets. Indeed, the thrombin induced activation of the AMPK in endothelial cells depends on the CaMKKβ. However, we found that CaMKK inhibition failed to inhibit thrombin-induced AMPK phosphorylation in platelets although it was effective in endothelial cells. Thrombin-induced activation of the AMPK was however paralleled by the phosphorylation of another kinase, LKB1 suggesting that it may act as an AMPKK under the conditions studied. The consequence of LKB1 phosphorylation for its activity is not clear. Indeed, LKB1 is assumed to be constitutively active and increases in cellular AMP levels were reported to stimulate AMPK activity by decreasing its dephosphorylation by the phosphatase PP2C. In endothelial cells however, stimuli such as fluid shear stress, peroxynitrite and the anti-diabetic drug metformin have all been reported to elicit the phosphorylation and increase the activity of LKB1. Thus, it now seems that phosphorylation does in fact play a role in LKB1 activation. Unfortunately, due to the lack of an appropriate LKB1 inhibitor and the inability to successfully apply small interfering RNA techniques to platelets, we were not able to provide direct evidence for the dependence of AMPK activity on the phosphorylation of LKB1. What are the events upstream of LKB1 phosphorylation? The phosphorylation of LKB1 as well as that of the AMPK were both sensitive to low concentrations of wortmannin indicating the upstream involvement of the PI3-K. Although LKB1 is not known to be a direct substrate of PI3-K, activation of LKB1 by the downstream effector kinase, Akt, may explain the effects observed. Indeed, LKB1 has been shown to be required for the Akt-dependent phosphorylation of pro-apoptotic proteins in a cancer cell line. However, the interaction between AMPK and Akt is both complex and controversial since Akt has been shown to inactivate AMPK in cardiac myocytes, while
the AMPK has been reported to negatively regulate Akt in breast cancer cells\textsuperscript{29} and in endothelial cells exposed to flow.\textsuperscript{30}

Platelet activation involves several initial steps, which include the release of Ca\textsuperscript{2+} followed by the activation of PKC leading to granule secretion, platelet aggregation and integrin αIβ3 phosphorylation or “inside-out activation”. Thereafter, the active integrin binds fibrinogen and initiates the outside-in signaling which is crucial for clot retraction and thrombus consolidation.\textsuperscript{31} The results of the present investigation indicate that the AMPK can influence processes involved in clot retraction. Indeed, although the inhibitors used affect the activity of several kinases, it was possible to show that the pharmacological inhibition of thrombin-induced clot retraction and β3 integrin phosphorylation observed in human platelets was also evident in platelets from AMPKα2\textsuperscript{-/-} mice. These \textit{in vitro} observations were supported by the finding that thrombus formation and stability were unaffected by the deletion of the AMPKα1 subunit but altered in AMPKα2\textsuperscript{-/-} mice in the \textit{in vivo} model of FeCl\textsubscript{3}-induced carotid artery injury. Taking all of this evidence together it seems safe to conclude that the AMPKα2 subunit appears to be more important than the α1 for the regulation of platelet function.

How could the AMPK affect platelet aggregation and clot retraction? One possibility is via interference with the αIβ3 integrin signaling. There is indirect evidence indicating such a link, as iodotubercidin has been reported to inhibit the phosphorylation of β3 integrin (on Thr753).\textsuperscript{32} However, attributing the latter effect to the AMPK would be controversial given that phosphorylation was induced by the phosphatase inhibitor calyculin A and a second AMPK inhibitor, 9-β-D-arabinofuranoside, was without effect. In the present study, we observed a clear kinase inhibitor-sensitive tyrosine phosphorylation of β3 integrin in human and murine platelets. Moreover, the thrombin-induced phosphorylation of β3 integrin was clearly inhibited in platelets from AMPKα2\textsuperscript{-/-} mice. These data indicated that AMPKα2 may modulate platelet function by modulating the activity of a β3 integrin phosphorylating tyrosine kinase. The most likely candidates to mediate the latter effect are the SFKs which are known to be activated by agonists such as thrombin,\textsuperscript{33} as well as by oxidative signaling events.\textsuperscript{34}
Several of this family of kinases, including Src and Fyn, can be detected in megakaryocytes and platelets and are thought to be involved in regulating platelet function, via binding to distinct sites on the cytoplasmic domain of β3 integrin. Although we saw no evidence for a link between AMPKα2 and Src, we found that AMPKα2 phosphorylated Fyn on Thr12, a residue reported to be associated with kinase activation.

In adipocytes, which express predominantly the AMPKα1 isoform, a link between Fyn and AMPK has already been suggested. However, in the latter study Fyn was proposed to act as a negative regulator of AMPK activation; most probably by phosphorylating LKB-1, thus preventing its translocation from the nucleus to the cytoplasm and decreasing AMPK activation. We also observed that Fyn phosphorylated the recombinant AMPKα1 and were unable to detect the phosphorylation of Fyn by AMPKα1 (authors unpublished observation) – findings that are in line with observations made in adipocytes. Moreover, our data also clearly indicate that AMPKα2 rather than AMPKα1 is linked with changes in platelet function, that AMPKα2 is able to phosphorylate Fyn on Thr12, and that the activated Fyn in turn phosphorylates platelet β3 integrin. This sequence of events can account for the attenuated clot retraction in human platelets treated with iodotubercidin or compound C as well as the formation of instable thrombi in the AMPKα2Δ mice. These observations are certainly of physiological and pathophysiological relevance as the abnormalities in platelet function described in the present study are similar to the platelet phenotype of Fyn-deficient mice.

Given that several diseases have been associated with altered AMPK activity (for review see Steinberg and Kemp) and that the AMPKα2 subunit has been linked to whole body insulin sensitivity, it will be interesting to determine whether or not changes in AMPK and/or Fyn activity can account for the altered platelet function observed in platelets from diabetic patients.
Acknowledgements

The authors are indebted to Isabel Winter, Katharina Bruch and Mechtild Piepenbrock for expert technical assistance, to Christian Gachet (Strasbourg, France) for technical help with the in vivo thrombosis model.

This study was supported by the Deutsche Forschungsgemeinschaft (SFB 815/A16 and the Exzellenzcluster 147 "Cardio-Pulmonary System") and by EICOSANOX, an integrated project supported by the European Community's sixth Framework Program (Contract N° LSHM-CT-2004-005033) and (LSHM-CT-2004-005272/ exogenesis).

Authorship contributions

V.R. designed and performed research, analyzed data and wrote the paper, J.I. and T.F. performed research and assisted with data analysis, B.F. optimized research materials, contributed to the analytical tools and coordinated animal supply, B.V. contributed vital new reagents, K.T.P. performed phospho-protein analyses and contributed to the experimental design and I.F. designed research and wrote the paper.

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

References


27. Soltys CL, Kovacic S, Dyck JRB. Activation of cardiac AMP-activated protein kinase (AMPK) by LKB1 expression or chemical hypoxia is blunted by increased Akt activity. *Am J Physiol Heart Circ Physiol* 2006;290(6):H2472-H2479.


Figure legends

Figure 1. **AMPKα subunit expression in human platelets and effect of thrombin.** (A) Expression of AMPKα1 and AMPKα2 in washed human platelets and the effect of thrombin (0.03 or 0.3 U/mL, 10 minutes) on AMPK phosphorylation of Thr172. (B) Effect of thrombin on the phosphorylation of ACC on Ser79. The bar graphs summarize data from 4 independent experiments; *P<0.05 and **P<0.01 versus solvent (Sol)-stimulated platelets.

Figure 2. **Parallel phosphorylation of LKB1 and AMPK in thrombin-stimulated human platelets.** (A) Concentration-dependent effect of thrombin on the phosphorylation of LKB1 on Thr189. (B) Effect of wortmannin (20 nmol/L) on the thrombin-induced phosphorylation of LKB1, AMPK and ACC. The bar graphs summarize data from 4 independent experiments; *P<0.05 and **P<0.01 versus solvent (Sol)-stimulated platelets.

Figure 3. **Effect of AMPK inhibition on platelet function.** Human platelets were pre-treated with either solvent (Sol), iodotubercidin (Iodo, 10 µmol/L) or compound C (Comp C, 10 µmol/L) and the effects of thrombin on (A) aggregation, (B) the peak increase in Ca²⁺ and (C) clot retraction were assessed. The graphs summarize data from 6 independent experiments; *P<0.05 and **P<0.01 versus solvent (CTL)-stimulated platelets.

Figure 4. **Role of AMPKα2 in clot retraction and thrombus stability.** (A) Expression of AMPKα subunits in murine platelets from wild-type (α1+/+, α2+/+), AMPKα1−/+ and AMPKα2−/+ mice and (B) thrombin-induced clot retraction in platelet-rich plasma from the same animals. (C) Representative images and summary of FeCl₃-induced thrombus formation in carotid arteries from AMPKα2+/+ and AMPKα2−/− mice. The arrows indicate emboli detaching from the thrombus. The graphs summarize data from 8 animals from each group; ###P<0.001 versus AMPKα2+/+.

Figure 5. **Role of AMPKα2 in the phosphorylation of β3 integrin.** (A) Effect of AMPK inhibition (Comp C, 10 µmol/L) on the thrombin-induced phosphorylation (p-Tyr747) of β3 integrin in washed human platelets. (B) Thrombin-induced phosphorylation of β3 integrin in
platelets from AMPKα2+/+, α2−/−, α1+/+ and α1−/− mice. The bar graphs summarize data from 6 different experiments; *P<0.05 and **P<0.01 versus solvent (Sol)-treated platelets.

**Figure 6. Role of AMPKα2 in the threonine phosphorylation of Fyn.** (A) Effect of SKF inhibition (PP2, concentration 30 nmol/L) on the thrombin-induced phosphorylation (p-Tyr747) of β3 integrin in washed human platelets. (B) In vitro kinase assays showing 32P incorporation into Fyn (left panel) and its threonine phosphorylation (p-Thr; right panel) in the presence of the AMPK. Experiments were performed in the absence and presence of PP2 iodotubercidin (Iodo) and compound C (CC). (C) Thrombin-induced phosphorylation of Fyn on Thr12 in platelets from AMPKα2+/+ and α2−/− mice. The bar graphs summarize data from 6 different experiments; *P<0.05 and **P<0.01 versus solvent (Sol)-treated platelets in the absence of thrombin.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

A

B

C

For personal use only.on September 24, 2017. For personal use only.
The AMP-activated protein kinase (AMPK) $\alpha_2$ subunit is involved in platelet signaling, clot retraction and thrombus stability

Voahanginirina Randriamboavonjy, Johann Isaak, Timo Frömel, Benoit Viollet, Beate Fisslthaler, Klaus T. Preissner and Ingrid Fleming