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ORIGINAL ARTICLE

MyosinIIa contractility is required for maintenance of platelet structure during spreading on collagen and contributes to thrombus stability

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Summary. *Background:* MyosinIIs are adenosine triphosphate-driven molecular motors that form part of a cell's contractile machinery. They are activated by phosphorylation of their light chains, by either activation of myosin light chain (MLC) kinase or inhibition of MLC phosphatase via Rho kinase (ROCK). MyosinIIa phosphorylation underlies platelet rounding and stress fiber formation. *Objective:* To identify the functional significance of myosinIIa in platelet spreading and thrombus formation on collagen using inhibitors of ROCK (Y27632) and myosinII (blebbistatin). *Results:* Stress fiber formation on collagen is inhibited by both Y27632 and blebbistatin. A substantial proportion of spread platelets generate internal holes or splits on collagen, presumably because of a reduction in contractile strength. Platelet integrity, however, is maintained. In an *in vitro* model, thrombus embolization on collagen is increased in the presence of Y27632 and blebbistatin at intermediate shear, leading to a reduction in platelet aggregate growth. Moreover, Y27632 causes a marked reduction in thrombus formation in an *in vivo* laser-injury model. *Conclusions:* MyosinIIa contractility is required for maintenance of platelet structure during spreading on collagen and contributes to thrombus stability.

Keywords: arterial thrombosis, cell signaling, MyosinII, platelet morphology, RhoA, Rho kinase.

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Introduction

Actin polymerization is regulated by Rho GTPases, with Cdc42, Rac and RhoA generating filopodia, lamellipodia and stress fibers, respectively [1,2]. These three structures are readily seen as platelets spread on multiple adhesive surfaces, including collagen and fibrinogen [3,4]. Cdc42 is activated during platelet spreading on collagen [4] and laminin [5], but there is as yet no direct evidence for a link between Cdc42 activation and filopodia formation in platelets. Rac is activated by multiple platelet receptor agonists, including adenosine diphosphate, collagen, thrombin and thromboxanes [6–10]. In addition, constitutively active Rac drives actin polymerization in permeabilized platelets [11]. Studies using mutant murine platelets have shown that Rac1 is essential for lamellipodia formation on fibrinogen, collagen, laminin and von Willebrand factor (VWF), both in the absence and presence of G protein-coupled receptor (GPCR) agonists, although aggregation to GPCR agonists is maintained. [12,13].

RhoA is activated by multiple adhesive surfaces and GPCR agonists, with activation mediated downstream of the heterotrimeric G protein, $G_{12/13}$ [10]. In turn, RhoA induces activation of Rho kinase (ROCK) and myosin light chain (MLC) phosphorylation, leading to stress fiber formation in adhered platelets and 'shape change' in an aggregometer [4,14,15]. In addition, MLC phosphorylation is regulated through Ca^{2+} activation of MLCkinase [14]. Inhibition of RhoA or ROCK using C3 exoenzyme and Y27632, respectively, has no effect on platelet aggregation to thrombin, collagen and ADP [3,16]. Similarly, RhoA or ROCK inhibition has a negligible or minor inhibitory effect on static adhesion to fibrinogen, VWF and collagen, or platelet capture by VWF under flow conditions [3,4,13,16]. RhoA and ROCK, however, do contribute to stable platelet adhesion on VWF under arterial rates of shear [16].

MyosinIIs are non-muscle forms of myosin involved in multiple cellular responses, including cytokinesis, phagocytosis and cell motility [17]. MyosinIIs consist of two heavy chains

and four light chains. MyosinII activation is mediated via MLC phosphorylation, either through an increase in MLC-kinase activity through Ca^{2+} elevation or by inactivation of myosin phosphatase downstream of ROCK. The importance of these two pathways in the regulation of MLC phosphorylation can be identified using the pharmacological inhibitors Y27632 for ROCK and BAPTA-AM for Ca^{2+}. In addition, myosinII can be directly inhibited using blebbistatin [18].

The major isoform of myosinII in platelets is myosinIIa [19]. Mutations of myosinIIa lead to the group of *MHY9* disorders, which include May-Hegglin anomaly, Sebastian, Fechtner and Epstein syndromes [20]. This group of disorders are caused by an autosomal dominant mutation in the gene encoding non-muscle myosin heavy chain IIa (NMMHC-IIA) and are associated with thrombocytopenia, giant platelets and a mild bleeding disorder, as well as different combinations of additional clinical features. Platelets from *MHY9* patients exhibit a defect in cytoskeletal reorganization in response to activation, leading to impairment in shape change and spreading. However, platelet aggregation is maintained [17].

The present study examines the role of ROCK and myosinII in mediating platelet spreading and thrombus formation on collagen. The results reveal an important role for myosinII in maintaining platelet integrity following adhesion to collagen and in stabilizing thrombus formation under flow conditions *in vitro* and *in vivo*. The defects in spreading and thrombus stability, in combination with the thrombocytopenia, could contribute to the mild bleeding diathesis that is observed in patients with the group of *MHY9* disorders.

Methods

Reagents

Y27632 and blebbistatin were obtained from Merck Biosciences (Nottingham, UK). The phosphospecific and pan antibodies to MLC were obtained from New England Biolabs (Ipswich, UK). The myosinIIa antibodies were from Covance (Harrogate, UK). Other materials were from previously described sources [12,21,22] or Sigma (Poole, UK).

Platelet preparation

Human venous blood was drawn by venipuncture as previously described [23]. Platelet-rich plasma was prepared by centrifugation of whole blood at 200 × *g* for 20 min. Platelets were isolated as previously reported and left for 30 min before experimentation [12]. Mouse platelets were prepared as previously reported [12].

Platelet aggregation and secretion

A quantity of 300 μL platelets (2×10^8 mL^{-1}) was incubated at 37 °C with DMSO (0.1%), Y27632 (50 μM), blebbistatin (100 μM) or cytochalasin D (10 μM) for 60 s. Chronolum reagent (20 μL) was added and platelets stirred for 60 s at 1200 rpm before addition of 1 or 10 μg mL^{-1} collagen. Aggregations were allowed to proceed for 3 min. The addition of an adenosine triphosphate (ATP) standard (2 nM) was used to measure ATP secretion. For determining efficacy of Y27632 *in vivo*, 300 μL of platelet-rich plasma were incubated with BAPTA-AM (40 μM) for 30 min, and integrillin (9 μM) for 2 min, before addition of 30 μg mL^{-1} collagen. All experiments were performed in a Born-lumiaggregometer (Chronolog, Haverton, USA).

Immunoblotting

Platelets (1×10^9 mL^{-1}) were incubated with integrillin (9 μM), and either DMSO (0.1%), Y27632 (3–50 μM) or blebbistatin (100 μM) for 2 min before stimulation with collagen (1–30 μg mL^{-1}) for 1–15 min. Platelets were lyzed and samples were run on sodium dodecylsulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes as previously reported [7]. Membranes were blotted for MLC (1:1000) or phosphorylated MLC (1:1000) before incubation with antirabbit secondary (1:10000). Blots were developed using ECL reagent (Amersham, Amersham, UK) and densitometry was conducted using Quantity One software (Bio-Rad, Hemel Hempstead, UK).

Platelet morphology

Coverslips were coated with matrix proteins as previously reported [12]. Platelets (2×10^7 mL^{-1}) were spread on collagen for 45 min at 37 °C in the presence of apyrase (2 U mL^{-1}), indomethacin (10 μM) and either DMSO (0.1%), Y27632 (50 μM) or blebbistatin (100 μM) before fixation with paraformaldehyde (3.7%). Platelets were then lyzed with Triton X-100 (0.2%) before staining with fluorescein isothicyanate (FITC)-phalloidin (2 μM) and an antibody to myosinIIa (1:100) for 60 min. Platelets were washed with PBS, before staining with Alexa-660 antirabbit secondary for 60 min. Images were analyzed using a DM IRE2 confocal microscope (Leica, Milton Keynes, UK) [12]. For platelet integrity studies, platelets were treated with Triton X-100 (0.2%) or PBS for 5 min before addition of FITC-phalloidin (2 μM) or $DiOC_6$ (2 μM), for 60 min at room temperature. Platelets were imaged using both Köhler illuminated Nomarski differential interference contrast (DIC) optics and fluorescent imaging with a Zeiss 63× oil immersion 1.40 NA plan-apochromat lens on a Zeiss Axiovert 200 M microscope. Platelet surface area was analyzed using IMAGEJ (NIH, Bethesda, USA) as previously reported [12].

F-actin assay

Filamentous actin was measured using a modified method of Machesky and Hall as previously described [24]. Platelets (5×10^8 mL^{-1}) were incubated with apyrase (2 U mL^{-1}), indomethacin (10 μM), and either DMSO (0.1%), Y27632 (50 μM) or blebbistatin (100 μM) for 2 min, before spreading for 45 min at 37 °C. A basal sample was removed while the

platelets were in solution and fixed as previously reported [12]. Coverslips were scraped three times to ensure removal of all platelets, and the sample was then rotated for 60 min in the dark. Platelets were pelleted by microcentrifugation for 2 min, washed with saponin washing buffer [12], and re-microcentrifuged. FITC-phalloidin was extracted with methanol and measured at 520 nm (emission wavelength), using 488 nm as excitation wavelength on a spectrofluorimeter. Alongside the F-actin assay, protein levels were measured with a Biorad Bradford assay kit to enable normalization.

Flow adhesion studies

Glass capillary tubes were coated with matrix proteins as previously reported [21]. Washed platelets (1×10^8 mL^{-1}) were incubated for 10 min at 37 °C with DMSO (0.1%), Y27632 (50 μM) or blebbistatin (100 μM). Red blood cells were isolated by washing with HEPES-buffered saline and three centrifugations at $2000 \times g$ for 10 min to remove plasma proteins. For real-time analysis, platelets were incubated with the fluorescent dye, $DIOC_6$ (2 μM). Washed platelets and red blood cells were reconstituted to a hematocrit of 50% and flowed over collagen for 3 min at 1000 s^{-1} or at 3000 s^{-1}. Real-time image sequences were recorded using a CoolSnap Camera (Photometrics, Huntington Beach, CA, USA) on a DM IRB microscope (Leica) [12,21]. Platelet thrombi were washed with buffer for 5 min and analyzed as previously reported [12]. For thrombus height, confocal stacks of adherent platelets were counted as previously reported [12].

Intravital microscopy

All procedures were undertaken with approval from the United Kingdom Home Office in accordance with the Animals (Scientific Procedures) Act of 1986. The cremaster muscle of male B57/Bl6 mice ($n = 3$) was exposed and prepared as previously described [12,26]. After exposing the cremaster muscle, arterial endothelial walls were subjected to damage by a nitrogen laser and the resultant thrombus monitored. Several thrombi were induced and recorded for each mouse before i.p. injection with Y27632 to give an estimated plasma concentration of 100 μM, as described in the results. A further five thrombi were induced in the presence of Y27632 within 5–30 min following injection. Images were captured with a high rate Senicam CCD camera (Cooke Corporation, Romulus, MI, USA) coupled to a Gen III image intensifier (Videoscope Int Ltd, Sterling, VA, USA) on an Olympus XX microscope. All images were analyzed using SLIDEBOOK 4.0 (Intelligent Imaging Innovations, Inc., Denver, CO, USA) as previously reported [12].

Analysis of results

Results are shown as mean ± SEM from at least three experiments. Statistical analysis was performed using ANOVA or Student's *t*-test.

Results

Collagen regulates myosin light chain phosphorylation through ROCK

Collagen (30 μg mL^{-1}) stimulates marked phosphorylation of MLC in a suspension of human platelets which peaks at 1 min and is reduced at 5 min and 15 min [Fig. 1 (i) and data not shown]. Phosphorylation is inhibited in a concentration-dependent manner by the ROCK inhibitor Y27632, with maximal inhibition observed at 30–50 μM [Fig. 1 (ii)]. Y27632 induced a much greater inhibition of phosphorylation of MLC following stimulation by collagen for 5 min (86 ± 6% inhibition) than after 1 min (44 ± 8%), consistent with previous reports that Ca^{2+} elevation induces rapid phosphorylation of MLC and that ROCK mediates sustained phosphorylation of MLC [14,25]. Similar observations were made with 1 and 10 μg mL^{-1} collagen (data not shown). In contrast MLC phosphorylation induced by collagen (1–30 μg mL^{-1}) over a time course of 1–15 min in the presence of the myosinII

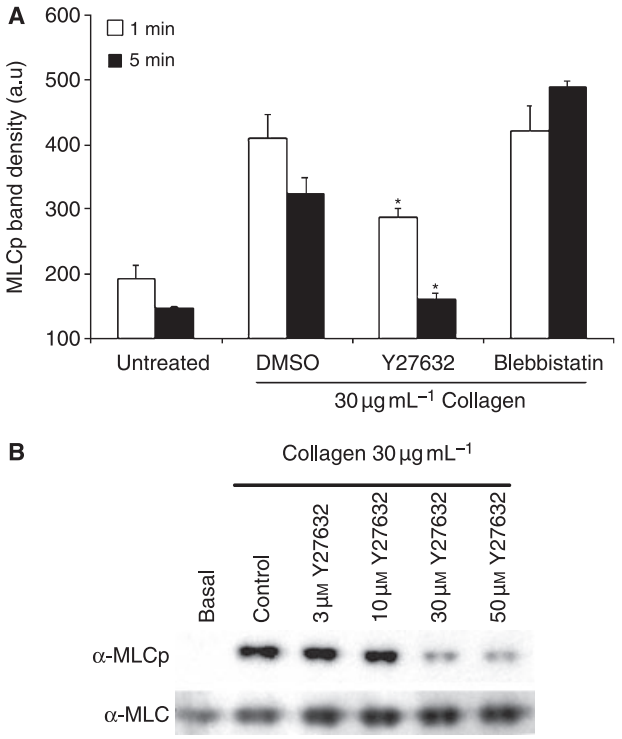

Fig. 1. Collagen stimulates phosphorylation of myosin light chain (MLC) through a Rho kinase-dependent pathway. Platelets were treated with DMSO (0.1%), Y27632 (50 μM) or blebbistatin (100 μM) for 2 min prior to stimulation with 30 μg mL^{-1} collagen for 1 min, or 5 min at 37 °C, and then lyzed. Samples were run on sodium dodecylsulfate–polyacrylamide gel electrophoresis gels, transferred to polyvinylidene difluoride (PVDF) and western blotted for MLC using antipan MLC and antiphospho MLC (MLCp) antibodies. (i) The intensity of MLCp western blots was quantified by densitometry, and the results are shown as the mean ± SEM of three experiments. *$P < 0.05$ relative to DMSO control. (ii) Example western blot of MLCp and MLC in the presence of Y27632 (3–50 μM) at 5 min. Images are representative of three experiments.

inhibitor, blebbistatin, used at a concentration that completely inhibits platelet stress fiber formation, was unaffected [Fig. 1 (i) and not shown]. This observation is consistent with previous reports that blebbistatin traps myosinII in a low affinity state for actin but does not inhibit MLC phosphorylation [18].

MyosinII is required for full aggregation and secretion to collagen

A series of experiments were designed to investigate the effect of Y27632, blebbistatin, and the actin polymerization inhibitor, cytochalasin D, on aggregation and secretion induced by collagen (1 and 10 μg mL^{-1}). The lower concentration collagen (1 μg mL^{-1}) stimulated partial aggregation and weak ATP secretion with a characteristic delay (Fig. 2A). At a tenfold higher concentration, collagen stimulated full aggregation and a more marked ATP secretion (Fig. 2A). In both cases, aggregation was preceded by shape change. Strikingly, treatment with Y27632 (50 μM) caused a more pronounced shape change response to both concentrations of collagen, although the rate and final extent of aggregation was unaffected when the increase in shape change was taken into account (Fig. 2A). Y27632 had no effect on secretion induced by either concentration of collagen (Fig. 2B). In comparison, blebbistatin (100 μM) caused a significant inhibition of aggregation and ATP secretion induced by both concentrations of collagen, while cytochalasin D (10 μM) had a slightly greater inhibitory effect on both responses (Fig. 2B). The results with cytochalasin D provide evidence for a contributory but non-essential role of actin assembly in platelet aggregation and secretion by collagen, whereas ROCK activity is not required for either response. It is unclear whether the inhibitory effect of blebbistatin is a consequence of its ability to produce a greater degree of inhibition of MLCkinase at early times of stimulation relative to Y27632 or through an additional action.

ROCK is required for stress fiber formation and structural integrity during spreading on collagen

Human platelets undergo sequential formation of filopodia, lamellipodia and stress fibers on collagen in the presence of inhibitors of the secondary feedback agonists, ADP and thromboxanes. After 45 min, the majority of platelets have formed extensive lamellipodia and stress fibers, and there is

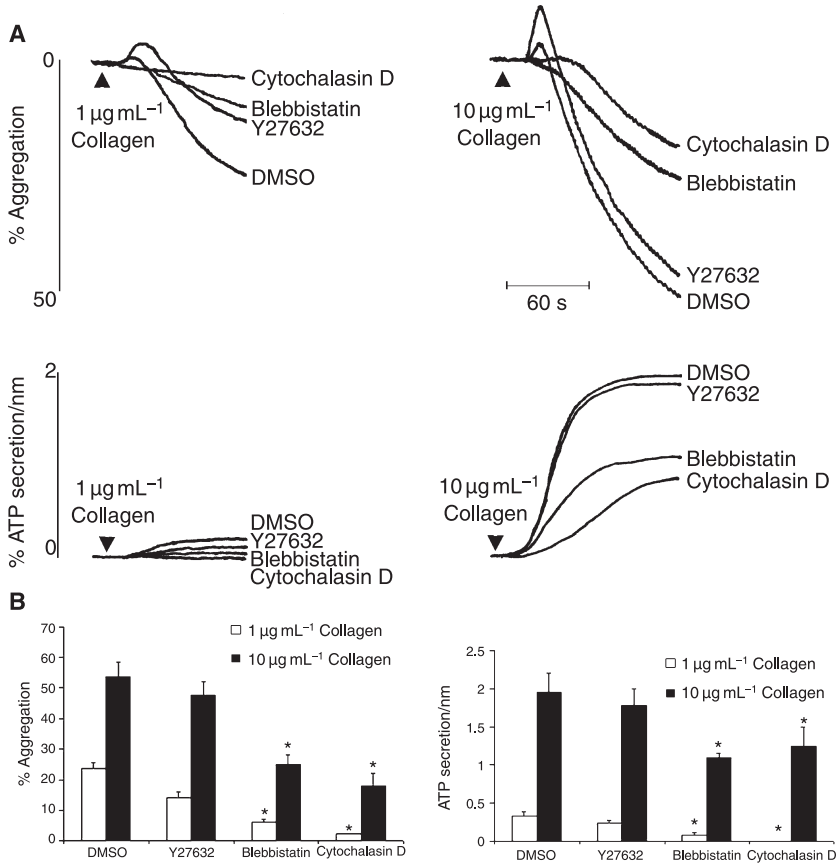

Fig. 2. The effect of inhibitors of actin remodeling on aggregation and secretion. Washed platelets were incubated with DMSO (0.1%), Y27632 (50 μM), blebbistatin (100 μM) or cytochalasin D (10 μM) for a minimum of 2 min prior to collagen stimulation. All experiments were performed in a Born-lumiaggregometer with stirring at 1200 × *g* and in the presence of chronolum to monitor adenosine triphosphate (ATP) secretion. (A) Aggregation and ATP secretion traces to 1 μg mL^{-1} collagen and 10 μg mL^{-1} collagen in the presence of inhibitors of actin remodeling. Collagen was added as indicated by the arrow. Results are representative of three experiments. (B) Maximum aggregation and ATP secretion for experiments described in (A). Results are mean ± SEM of three experiments. *$P < 0.05$ relative to the control.

extensive colocalization of actin with myosinIIa with the exception of a distinct outer ring of actin (Fig. 3A). The ROCK inhibitor Y27632 inhibited stress fiber formation on collagen and markedly reduced the colocalization of actin and myosinIIa (Fig. 3A). However, there was no significant change in platelet surface area, at either 22.5 or 45 min, in the presence of Y27632 and blebbistatin in comparison with the DMSO control (Fig. 3B and data not shown), demonstrating that lamellipodia formation was unaltered. Strikingly, the presence of internal holes and splits could be seen in 10–15% of the platelets that had spread on collagen in the presence of Y27632 and blebbistation, whereas similar structures were present in $<0.5\%$ of control cells (Fig. 3-A,C). We have termed these structures fenestrations (i.e. windows). To investigate whether the fenestrations are due to an increase in permeability of the plasma membrane, platelets were fixed and incubated with FITC-phalloidin in the absence or presence of Triton X-100 (to induce permeabilization). Staining of the actin cytoskeleton by FITC-phalloidin was only observed in the presence of Triton X-100, demonstrating that membrane integrity is maintained in the presence of Y27632 or blebbistatin (Fig. 4B). Consistent with this, the fenestrations were observed to undergo continuous movement throughout the recording (Fig. 4A). Together, these studies demonstrate a critical role for ROCK and myosinIIa in stress fiber formation in spread platelets. Furthermore, following inhibition of ROCK or myosinIIa, a significant number of platelets exhibit fenestrations, which indicates a structural weakening of the actin cytoskeleton.

Actin polymerization induced by collagen is not altered in the presence of Y27632 and blebbistatin

The level of F-actin was measured in platelets that had been allowed to spread on collagen-coated coverslips to investigate whether the loss of stress fiber formation and appearance of fenestrations was associated with a change in the level of actin polymerization. There was no significant difference in the level of F-actin in spread platelets treated with either Y27632 or blebbistatin in comparison with the DMSO control (Table 1).

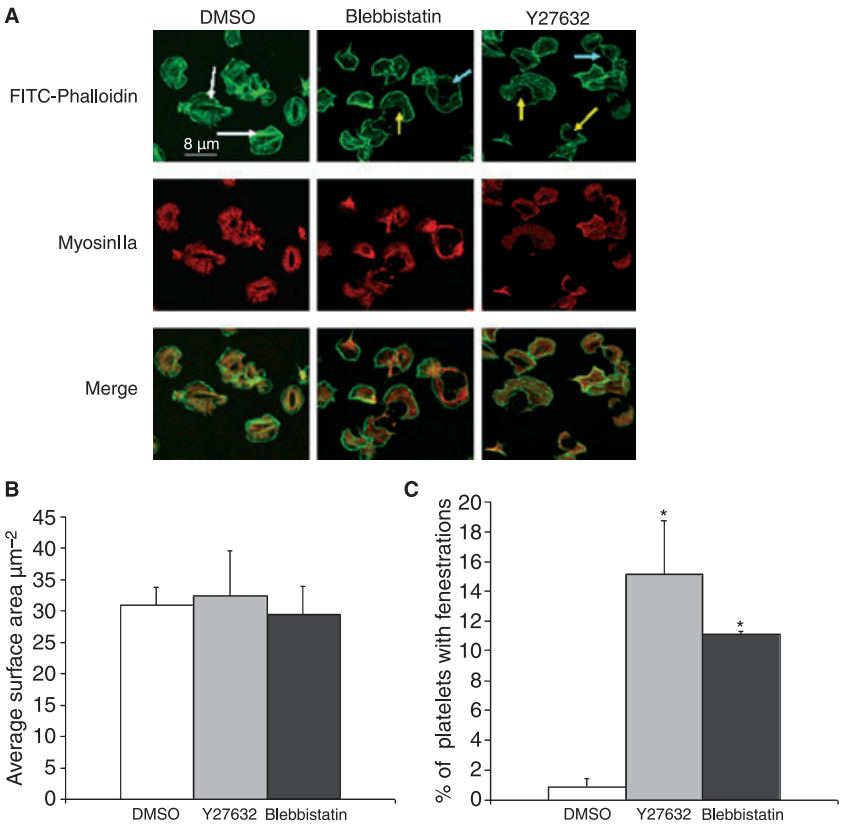

Fig. 3. Y27632 and blebbistatin inhibit stress fiber formation and generate fenestrations on collagen. Platelets were allowed to spread on collagen-coated coverslips for 45 min at 37 °C in the presence of apyrase (2 U mL^{-1}) and indomethacin (10 μM), and either DMSO (0.1%), Y27632 (50 μM) or blebbistatin (100 μM), before fixation with paraformaldehyde (3.7%). (A) Platelets were lyzed with Triton X-100 (0.2%) and then stained with fluorescein isothicyanate-phalloidin (2 μM), and a myosinIIa antibody. The overlay shows the comparison of localization of actin and myosinIIa. The white arrow illustrates stress fibers, and the yellow and blue arrows identify sites of splitting and appearance of holes within the platelet, respectively (collectively fenestrations). Images are representative of three experiments. (B) Differential interference contrast microscope images were recorded and platelet surface area was quantified. Results represent the mean ± SEM of a minimum of 100 platelets from one experiment that is representative of three. (C) Platelets were lyzed and stained as described in part (A). The percentage of platelets with fenestrations was counted (mean ± SEM). A minimum of 100 platelets was analyzed per experiment and $n = 3$. $^*P < 0.05$ relative to the control.

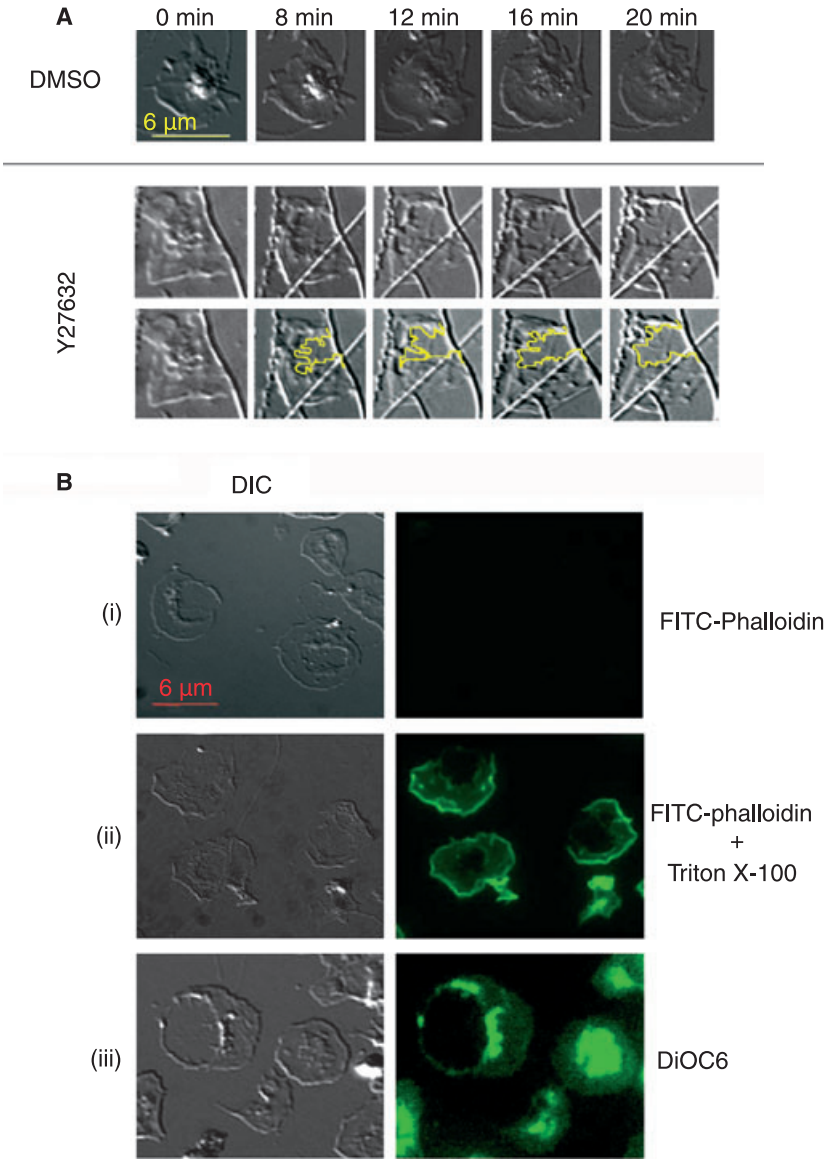

Fig. 4. Demonstration of dynamic fenestration formation and maintenance of platelet integrity. (A) Platelets were allowed to spread on collagen-coated coverslips in the presence of apyrase (2 U mL^{-1}), indomethacin (10 μM) and either DMSO (0.1%) or Y27632 (50 μM). Spreading was monitored using differential interference contrast (DIC) microscopy. The images illustrate the dynamic movement of the membrane, with the yellow lines following the cell membrane of the platelet. The results are illustrative of three experiments. (B) Platelets were allowed to spread on collagen-coated coverslips for 45 min in the presence of apyrase (2 U mL^{-1}), indomethacin (10 μM), and either DMSO (0.1%) or Y27632 (50 μM) at 37 °C before fixation with paraformaldehyde. Platelets were stained with (i) fluorescein isothicyanate (FITC)-phalloidin (2 μM), (ii) FITC-phalloidin (2 μM) after treatment with Triton X-100 (0.2%), (iii) $DiOC_6$ (2 μM), for 60 min. Images were taken using DIC and fluorescent optics. The results are representative of three experiments.

Table 1 Actin polymerization induced by collagen is not altered in the presence of Y27632 and blebbistatin

Treatment	Fold increase in F-actin in comparison with basal
DMSO	1.81 ± 0.03
Y27632 (50 μM)	2.04 ± 0.14
Blebbistatin (100 μM)	2.04 ± 0.36

Platelets (2×10^8 mL^{-1}) were incubated with DMSO (0.1%), Y27632 (50 μM) or blebbistatin (100 μM) for 2 min before spreading on collagen-coated plates for 45 min. F-actin levels were measured and compared with that of a quantity matched basal platelet sample.

Stress fiber formation is required for aggregate integrity under flow

The significance of inhibition of stress fiber formation on platelet aggregate formation on collagen under shear conditions was examined. These studies were performed at intermediate and high shear rates of 1000 and 3000 s^{-1} that are typically found in the arteriolar system, using washed platelets labelled with the fluorescent dye $DiOC_6$. Washed platelets were used because of extensive protein binding of blebbistatin, rendering it ineffective in plasma (unpubl. obs.). The experiments were performed in the presence of red

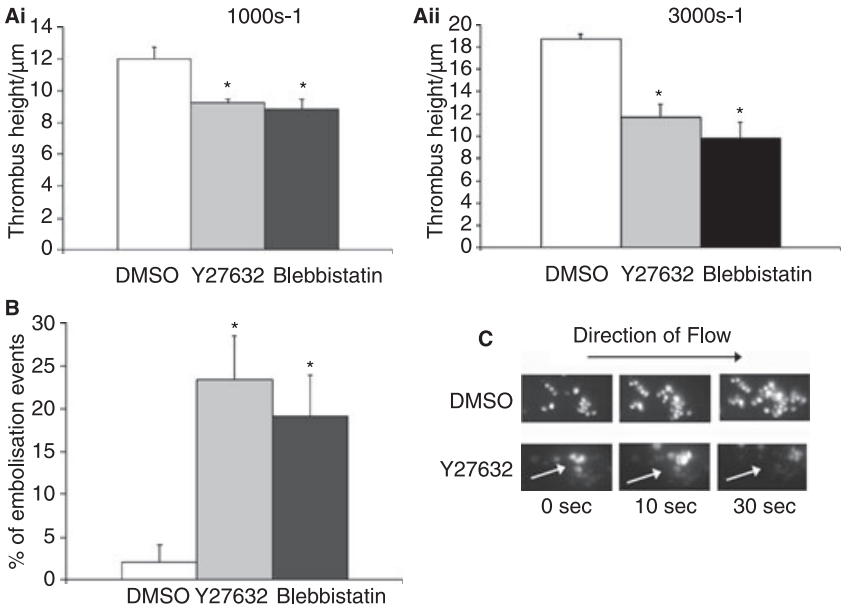

Fig. 5. Y27632 and blebbistatin inhibit platelet aggregate formation on collagen. Washed platelets were reconstituted with red blood cells and incubated with DMSO (0.1%), Y27632 (50 μM) or blebbistatin (100 μM) for 10 min at 37ºC. Platelets were then flowed over collagen for 3 min at either 1000 or 3000 s^{-1}, before washing with Tyrode's buffer for 5 min. Thrombi were fixed using paraformaldehyde (3.7%) for 30 min and incubated with $DiOC_6$ (2 μM) overnight at room temperature. (A) Thrombus height at (i) 1000 s^{-1} and (ii) 3000 s^{-1} was calculated using confocal microscopy. (B) Thrombus formation at 1000 s^{-1} was monitored by fluorescence microscopy in real time and the rate of embolization was measured by analysis of real-time courses. Embolization was defined as movement of platelets from the original point of contact. (C) An example recording illustrating thrombus embolization in the presence of Y27632 (50 μM). The arrow at 0 s illustrates the build-up of a small thrombus, which then embolizes in the direction of flow. Images are representative of three experiments. Results are shown as mean ± SEM of three experiments. $*P < 0.05$ relative to the control.

blood cells to ensure flow of platelets at the edge of the chamber.

Inhibition of ROCK and myosinII caused a significant reduction in mean aggregate height at both shear rates, although the effect was more pronounced at 3000 s^{-1} (Fig. 5A,B). The relative small nature of the inhibition at 1000 s^{-1} indicates that aggregate formation is largely independent of ROCK and myosinII at intermediate shear but that this becomes more dependent on the actin cytoskeleton as shear is increased. The reduction in thrombus height is mirrored by an increase in the frequency of embolization relative to DMSO-treated controls (Fig. 5C). In contrast, neither inhibitor had a significant effect on surface coverage (data not shown). Examination of real-time video recordings demonstrates that platelet capture and aggregate formation is similar in the presence of Y27632 and blebbistatin, suggesting that the increase in embolization underlies the decrease in aggregate formation (Fig. 5D). Together, these results indicate that blockage of stress fiber formation leads to aggregate instability on collagen, although platelet capture is maintained.

Inhibition of ROCK with Y27632 inhibits thrombus formation in vivo

Intravital microscopy was undertaken to investigate the functional significance of inhibition of ROCK activation using Y27632. Similar studies could not be performed using blebbistatin because of extensive protein binding in plasma. Initially, a series of experiments were performed to establish the degree of protein binding of Y27632 in plasmas using platelet shape change as a readout (Fig. 6). These studies were performed in BAPTA-loaded platelets to prevent MLCkinase activation by Ca^{2+} elevation. Y27632 (10 and 100 μM) caused partial and complete block of shape change to the PAR-4 specific peptide, thrombin related activating peptide (TRAP), respectively (Fig. 6A). Similar results were obtained in washed platelets (Fig. 6B), demonstrating that Y27632 is minimally protein bound in plasma. This observation was used to estimate the dose of Y27632 to induce inhibition of ROCK following i.p. injection in mice, on the assumption that Y27632 distributes freely in the aqueous compartments in the cell. *Ex vivo* analysis of platelet shape change in BAPTA-loaded platelets revealed that 2.37 mg kg^{-1}, and 23.7 mg kg^{-1} induce partial and full blockade of shape change to collagen (Fig. 6B).

Thrombus formation *in vivo* was analyzed by inducing damage to the endothelial layer using a nitrogen dye laser and monitoring thrombus formation in real time using a non-blocking FITC-conjugated antibody that recognizes the major platelet integrin αIIbβ3 [26]. Thrombus formation induced by laser injury was measured before and after injection of Y27632 (23.7 mg kg^{-1}). All experimentation was carried out between 5 and 30 min following treatment with Y27632. Figure 7A illustrates the steady build-up of platelets into a large, stable thrombus under control conditions with only minimal embolization. In comparison, following laser injury in the presence of Y27632, platelets were unable to form large stable thrombi and

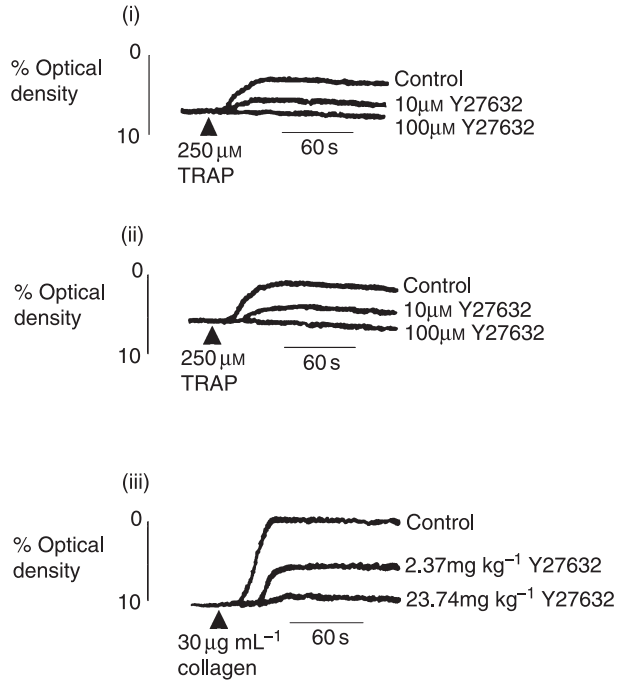

Fig. 6. Inhibition of platelet shape change after administration of Y27632 to washed platelets and platelet-rich plasma. (i) Washed platelets were treated with BAPTA-AM (40 μM) for 30 min to block the regulation of shape change downstream of Ca^{2+}, and Y27632 (10–50 μM) for 2 min, before stimulation by thrombin related activating peptide (TRAP) (250 μM) (indicated by the arrow). Aggregation was inhibited with αIIbβ3 blocker, integrillin (9 μM). (ii) Platelets were stimulated in platelet-rich-plasma by TRAP (250 μM) (indicated by black arrowhead) under the same conditions as for (i). (iii) Mice injected with saline, 2.37, or 23.74 mg kg^{-1} Y27632 for 30 min before exsanguination. Platelet-rich plasma was prepared and loaded with BAPTA-AM for 30 min before stimulation with 30 μg mL^{-1} collagen (indicated by arrow). All traces are representative of three experiments.

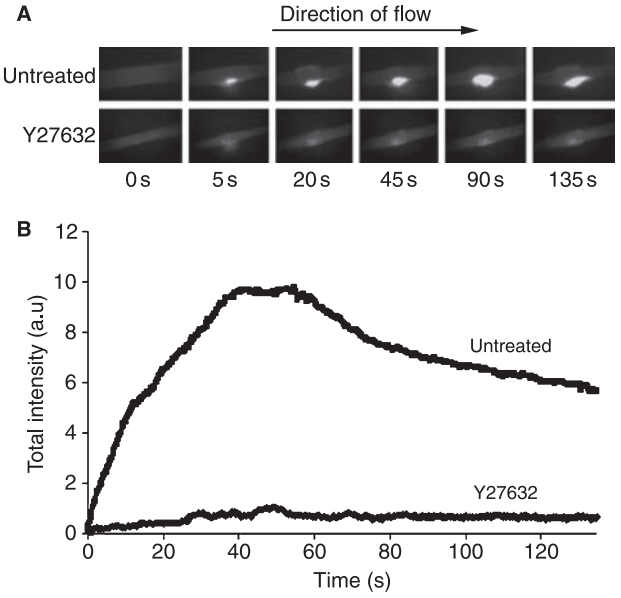

Fig. 7. Decreased thrombus formation in Y27632-treated mice after laser-induced injury. The effect of Y27632 (23.74 mg kg^{-1}) on thrombus formation was monitored within the same animal, with an average of five thrombi produced both before and after administration of Y27632 (with recordings between 5 and 30 min following Y27632 injection). (A) An example recording illustrating thrombus embolization in control and Y27632-treated animals (100 μM). (B) Mean integrated fluorescence of five thrombi pre- and post-Y27632 injection. The data are representative of experiments on three animals.

exhibited rapid embolization. The overall extent of thrombus formation was significantly reduced following treatment with Y27632 (Fig. 7B). These results demonstrate that ROCK is required for stable thrombus formation at arterial rates of shear *in vivo*.

Discussion

The present study demonstrates that ROCK and myosinII play critical roles in maintaining platelet morphology and thrombus stability under *in vitro* and *in vivo* conditions. Inhibition of ROCK and myosinII significantly affects platelet morphology during platelet spreading on collagen, as demonstrated by formation of internal holes and splits (fenestrations) and blockade of stress fiber formation. These changes may therefore underlie the increase in embolization observed under *in vitro* and *in vivo* conditions, possibly as a consequence of loss of structural integrity, although there is no direct evidence that this is the case. The increase in embolization is, however, very likely to contribute to the bleeding diathesis that is seen in patients with the *MHY9* group of disorders caused by mutation of non-muscle myosin heavy chain IIa, along with the associated thrombocytopenia.

The present results agree with earlier studies that platelet spreading on fibrinogen and collagen is unaltered by inhibition of ROCK [3,4]. However, this is the first study to report fenestration formation in platelets in the presence of Y27632, thereby revealing a critical role for ROCK in maintaining platelet morphology. Significantly, a similar set of observations was obtained with blebbistatin. Real-time video microscopy demonstrated that fenestration formation in the presence of Y27632 and blebbistatin was not simply because of a loss of platelet integrity, as the change in morphology was continuous, and moreover actin fibers could not be stained with the membrane-impermeable toxin, phalloidin, without prior permeabilization. Fenestration formation was not due to a reduction in F-actin production. The present results are similar to the effect of RhoA inhibition by C3 toxin in Swiss 3T3 cells [27]. In this study, the presence of fenestrations is readily apparent in C3 toxin-treated but not in control fibroblasts that have been allowed to undergo spreading.

The possibility that the reduction in thrombus stability is due to the loss of structural integrity is consistent with the proposal by Canobbio *et al.* [17] that GTPase incorporation into the cytoskeleton is vital for late events of thrombus formation. On the other hand, thrombus formation is regulated by multiple factors and it is possible that additional actions of ROCK and myosinII, rather than fenestration formation or stress fiber

inhibition, contribute to this instability. For example, ROCK inhibition by Y27632 has been reported to reduce aggregate formation in a cone-and-plate visocometer and adhesion of platelets to VWF within a high shear environment [16]. In addition, it is possible that the inhibitors have other inhibitory actions that have contributed to the reduction in thrombus stability. For example, blebbistatin causes a partial reduction in secretion and aggregation but this cannot explain the reduction in thrombus stability as Y27632 had a negligible effect on these two responses. On the other hand, ROCK has been implicated in a number of other pathways that could have potentially contributed to the thrombus stability that was seen *in vivo*, including expression of tissue factor and pro-coagulant activity [28,29]. Pro-coagulant activity is known to play an important role in the laser-induced injury model alongside that of the thrombin receptor, PAR4, and the collagen receptor, GPVI [30,31]. Therefore the contribution of coagulation to thrombus formation after laser-induced injury may be reduced in the presence of Y27632. It is possible that the contribution of ROCK to contraction of vascular smooth muscle, reduction in blood pressure, nitric oxide synthesis (eNOS), and maintenance of vascular tone, effects thrombus formation *in vivo* [32]. Importantly, however, inhibition of these responses is unable to account for the increase in embolization that is seen under *in vitro* flow conditions that are seen in the presence of Y27632.

The effect of Y27632 *in vivo* is further complicated by Y27632 affecting other cells in the bloodstream, including endothelial cells. ROCK inhibition is associated with an increase in eNOS from endothelial cells, which could drive vasodilation and reduce platelet activation [33]. ROCK inhibition also leads to a reduction in migration, cell adhesion and cell permeability. This raises the possibility that the reduction in thrombus formation that we have observed *in vivo* may not be due to myosinII inhibition, as we do not have equivalent results for blebbistatin.

The present results can be compared with the recent observations of McCarty *et al.* [12], who demonstrated increased platelet embolization on immobilized collagen or immobilized VWF/thrombin in $Rac1^{-/-}$ platelets. $Rac1^{-/-}$ platelets cannot form lamellipodia and therefore also have altered stress fiber formation, which could have contributed to the thrombus instability observed in the absence of the small G protein. Therefore, our results are consistent with stable thrombus formation requiring both lamellipodia formation and stress fiber generation, and that Rac and Rho co-ordinate with each other to mediate stable thrombus formation *in vivo* under flow.

In conclusion, we have demonstrated a requirement for ROCK and myosinII in maintaining the platelet cytoskeleton and thrombus stability on collagen at arterial rates of flow. These studies further underscore the role of the cytoskeleton in contributing to thrombus formation *in vivo*. Additionally, this study emphasizes the potential of a ROCK inhibitor in the treatment of hypertension and its associated thrombotic complications, as the inhibitor would have both an anti-hypertensive and antithrombotic action.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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