Flow-Induced PRotusions (FLIPRs): A Platelet-Derived Platform for the Retrieval of Microparticles by Monocytes and Neutrophils

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ABSTRACT

Rationale: Platelets are the most important cells in the primary prevention of blood loss after injury. In addition, platelets are at the interface between circulating leukocytes and the (sub)endothelium regulating inflammatory responses.

Objective: Study the dynamic process that leads to the formation of procoagulant and pro-inflammatory platelets under physiological flow.

Methods and Results: In the present study we describe the formation of extremely long, negatively charged membrane strands that emerge from platelets adhered under flow. These Flow-Induced PRotusions (FLIPRs) are formed in vitro on different physiological substrates and are also detected in vivo in a mouse carotid injury model. FLIPRs are formed downstream the adherent and activated platelets, and reach lengths up to 250 μm. FLIPR formation is shear dependent, and requires cyclophilin D, calpain and Rac1 activation. It is accompanied by a disassembly of the F-actin and microtubule organization. Monocytes and neutrophils roll over FLIPRs in a P-selectin/PSGL-1 dependent manner, retrieving fragments of FLIPRs as microparticles on their surface. Consequently, monocytes and neutrophils become activated, as demonstrated by increased CD11b expression and L-selectin shedding.

Conclusions: Formation of long platelet membrane extensions, such as presented in our flow model, may pave the way to generate an increased membrane surface for interaction with monocytes and neutrophils. Our study provides a mechanistic model for platelet membrane transfer and the generation of monocyte/neutrophil-microparticle complexes. We propose that the formation of FLIPRs in vivo contributes to the well-established pro-inflammatory function of platelets and platelet-derived microparticles.

Keywords: Platelet adhesion, flow, FLIPRs, inflammatory response, monocyte, microvesicles

Nonstandard Abbreviations and Acronyms:
FLIPRs  Flow-Induced PRotusions
PS  Phosphatidylserine
SOCE  Store operated calcium entry
MPTP  Mitochondrial permeability transition pore
TRPC  Transient receptor potential C
PSGL-1  P-selectin glycoprotein ligand-1
PDMS  Polydimethylsiloxane
CRP  Collagen related peptide
DTT  Dithiothreitol
SDS-PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis
PRP  Platelet rich plasma
WP  Washed platelets
GPIb  Glycoprotein Ib
PF4  Platelet factor 4
2-APB  2-aminoethoxydiphenylborate
MP  Microparticle
OCS  Open canalicular system
COAT  Collagen- and thrombin-activated
SCIP  sustained calcium-induced platelet
RICM  Reflection Interference Contrast Microscopy
INTRODUCTION

Platelets play a prominent role in the human primary hemostatic response. During thrombus formation, platelets have two main functions; 1) initial adhesion to exposed subendothelium, as may occur during injury or rupture of atherosclerotic lesions, 2) recruitment of circulating platelets in an integrin αIIbβ3-dependent manner, expanding thrombus volume. Activation of platelets after exposure to strong agonists induces the expression of phosphatidylserine (PS) on the platelet surface, required for the accumulation of clotting factors. Procoagulant platelets form PS-exposing microparticles and further develop into balloon-shaped platelets capable of supporting fibrin formation. The formation of procoagulant platelets is triggered by a strong and sustained rise in the cytosolic calcium concentration. Upon depletion of the intracellular calcium stores, store-operated calcium entry (SOCE) is activated, leading to an influx of calcium. Additionally, store-independent calcium entry via nonselective cation channels of the transient receptor potential C (TRPC) family can be activated leading to increased PS-exposure and procoagulant activity. Mitochondrial events have been implicated as important determinants of platelet procoagulant activity. Calcium transport from the mitochondria is regulated via the formation of the mitochondrial permeability transition pore (MPTP), a nonselective multiprotein pore that spans the inner mitochondrial membrane. This process is regulated by the peptidylprolyl isomerase cyclophilin D and results in a rapid loss of mitochondrial transmembrane potential causing high levels of PS exposure.

Besides the well-known contribution to thrombus formation, platelets have been implicated in inflammatory diseases, mainly through their capacity to directly interact with leukocytes. Activated platelets can bind leukocytes and attract them towards the arterial wall, thereby promoting inflammatory responses such as atherosclerosis, venous thrombosis and arthritis. Furthermore, activated platelets can bind to both monocytes and neutrophils, forming a circulating complex. Increased numbers of such complexes are reported in patients after percutaneous coronary intervention, acute myocardial infarction, stable coronary artery disease and ischemic stroke.

The dynamic process that leads to the formation of procoagulant and pro-inflammatory platelets under physiological flow has never been visualized. In the present study, we show the timed formation of FLow-Induced PRotusions, termed FLIPRs. These are extremely long, negatively charged membrane strands that emerge from spread platelets after activation. This consequently results in the formation of microparticles and balloon-shaped platelets. FLIPRs are formed via cyclophilin D dependent calcium transport, and require calpain and Rac1 activation. Circulating platelets do not interact with pre-existing FLIPRs, suggesting reduced adhesive properties. Monocytes and neutrophils, on the other hand, preferentially roll over FLIPRs and FLIPR-forming platelets in a P-selectin - PSGL-1 depending fashion. This interaction leads to the retrieval of FLIPR fragments as microparticles on their surface and leads to their activation.

METHODS

Detailed methods are available as an online supplement.

Blood collection.
Blood was collected from healthy volunteers and anti-coagulated with 10% sodium citrate (3.2% w.v.). Platelet rich plasma (PRP) and washed platelets were prepared as described previously.

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Real-time perfusion studies.
Glass coverslips were placed in a polydimethylsiloxane (PDMS) perfusion chamber coated with fibrinogen (100 µg/mL). Washed platelets were perfused in a calcium rich buffer at a shear rate of 300 sec⁻¹ and activated by perfusing collagen related peptide (CRP, 500 ng/mL) over the platelets after 15 minutes of perfusion.

Microparticle isolation.
Washed platelets were perfused in a calcium rich buffer over fibrinogen and activated with 500 ng/mL CRP. Flowthroughs were collected and centrifuged twice at 1500xg for 15 minutes in the presence of PGI₂ and 20 mM EDTA. For FACS analysis, the supernatant containing microparticles was incubated with antibodies against GPIb and lactadherin. For westernblots, the supernatant was centrifuged at 20,000xg for 1 hour, and the microparticle-containing pellet resuspended in sample buffer in the presence of dithiothreitol (DTT). Proteins were separated by SDS-PAGE and stained with anti-GPIb (SZ-2).

Monocyte and neutrophil perfusions.
Monocytes were isolated using CD14⁺ magnetic beads from the mononuclear cell fraction obtained via Ficoll separation. Neutrophils were isolated with a 6% Dextran/0.9% NaCl solution followed by a Ficoll. Washed platelets were perfused for 15 minutes followed by activation with CRP for 5 minutes. Monocytes and neutrophils were then perfused, and rolling and adhesion were studied during real-time perfusion. The flowthroughs during these perfusions were captured and stained with CD14 (monocytes), CD66b (neutrophils), GPIb, CD11b or CD62L.

In vivo FLIPR formation.
A vascular injury model was used in mice using 7.5% FeCl₃. Arteries were fixed 2 min after induction of the lesion and processed for scanning electron microscopy (SEM) analysis.

RESULTS

Formation of shear-induced platelet membrane protrusions.
Washed platelets were perfused in a calcium rich buffer (1 mM) through a perfusion chamber coated with fibrinogen or fibronectin at a low arterial shear rate of 300 sec⁻¹. Platelets adhered readily to the immobilized proteins and became fully spread. CRP (500 ng/mL) was subsequently perfused to further activate the adhered platelets via interaction with glycoprotein VI (GPVI). Upon addition of CRP, spread platelets started forming long membrane protrusions (Figure 1A, supplemental movie I). These FLow Induced PROtrusions (FLIPRs) derive from the luminal platelet membrane (Supplemental movie II) and are formed in the direction of the flow (arrowheads in Figure 1A,B). The length of the FLIPRs was variable, FLIPRs up to 250 µm were frequently observed. Their initiation and full extension is usually within seconds, but could occasionally take up to 5 minutes. FLIPR formation occurred on 19.5 ± 4.1% of the spread platelets after activation with CRP and was generally detected within 5 minutes after CRP addition. A low extent of FLIPR formation was also observed upon perfusion of PRP, in the absence of CRP on all physiologic substrates, including fibrinogen, fibronectin, collagen peptides GFOGER + CRP-XL and VWF (Figure 1B). However, all these substrates appeared less potent in activating platelets, and hence activation with strong agonists appears to be required to reach a high percentage of FLIPR formation. Platelets with a FLIPR were fully activated, as determined by expression of P-selectin, and decreased cellular content of α-granule marker PF4 (Figure 1C). The membrane-bound activation marker P-selectin was also present on the FLIPR membrane (Figure 1E,F), indicating fusion and membrane delivery from alpha granules. To test the possibility that FLIPRs have a procoagulant nature, we analyzed the interaction with annexin A5 to study the potential exposure of negatively charged phospholipids.
FLIPRs and accessory platelets readily bound annexin A5 (Figure 1D), whereas platelets without FLIPRs were negative. These results indicate that FLIPR-forming platelets and their membrane extensions have a procoagulant potential.

**FLIPR formation requires membranes of the open canalicular system (OCS) and disruption of the cytoskeleton.**

FLIPR elongation was accompanied by major changes in platelet morphology. These included a change in shape and size (Figure 2A-C, supplemental movie III) of the adherent platelet, frequently with an almost complete loss of the platelet membrane integrity and a disassembly of the underlying cytoskeleton (arrowheads in Figure 2C”). The formation of long membrane protrusions requires a high amount of platelet membrane, presumably generated by the membrane reservoir of the open canalicular system (OCS). Since these internal stores harbor GPIb, we determined the mean GPIb fluorescent intensity in protruding and non-protruding cells spread on fibrinogen, as a measurement for OCS membrane consumption through FLIPR formation. The mean GPIb fluorescent intensity appeared more than 50% decreased in platelets that form FLIPRs as compared to platelets without a FLIPR (Figure 2E), indicating significant membrane loss from adherent platelets at the expense of forming protrusions. Fluorescent staining of the actin cytoskeleton using phalloidin-FITC revealed a characteristic filamentous staining pattern in intact spread platelets (Figure 2D, left panel). In contrast, FLIPR-forming platelets showed substantial disorganized actin architecture (Figure 2D, top right panel). Actin patches were frequently decorating the FLIPR membrane in a bead-like fashion (arrowheads in Figure 2D, bottom right panel). In non-FLIPR-forming spread platelets, microtubules were arranged in a characteristic circular fashion (Figure 2F, left panel) and became disorganized in platelets forming protrusions (arrow in Figure 2F right panel). Whole mount electron microscopy analysis revealed thin elongated membrane protrusions extending down-stream of the spread platelets (Figures 2G,H). These membranes expose frequently thicker regions particularly at their down-stream ends that contain electron-dense material, presumably actin (arrowheads in Figure 2G,H). These results indicate that platelet FLIPR formation requires both OCS consumption and the loss of F-actin and microtubule integrity. The final end stage morphology of the adhered platelet often resembled that of balloon-shaped ghosts (arrow in Figure 2F, right panel).

**Store-operated and store-independent calcium entry are involved in FLIPR formation.**

Flow experiments over fibrinogen-coated surfaces using citrated platelet rich plasma resulted in low FLIPR formation (3.1 ± 0.3%). EGTA completely blocked the formation of FLIPRs (Figure 3A). Also, washed platelets perfused over fibrinogen did not form FLIPRs in the absence of extracellular calcium. Addition of CRP resulted in extensive FLIPR formation on fibrinogen in the presence of calcium (19.5 ± 4.1% of platelets, Figure 3C). No FLIPR formation was observed when CRP was added to a calcium-free buffer. The influx of calcium was visualized using Oregon green labeling. CRP stimulation of spread platelets in the presence of calcium, revealed a distinct calcium peak prior to the formation of FLIPRs (arrow in Figure 3B), indicating an influx of calcium into the cytoplasm. (Figure 3C). Addition of thrombin (IIa, 0.5 U/mL) resulted in 17.5 ± 5.6% of FLIPR formation, whereas the simultaneous addition of CRP and thrombin resulted in high (79.4 ± 5.6%) FLIPR formation within 2 minutes (Figure 3C). After 5 minutes of stimulation, these FLIPRs frequently severed, leaving behind typical balloon-shaped platelet remnants (Figure 3C).

We next investigated the role of store-operated calcium entry (SOCE) and store-independent calcium entry on FLIPR formation. First, 2-aminoethoxydiphenylborate (2-APB) was added to block SOCE(19). Addition of 2-APB after the spreading of platelets effectively prevents FLIPR formation upon addition of CRP (2.0 ± 1.1% of platelets, Figure 3D). Furthermore, induction of direct calcium influx from intracellular stores using thapsigargin(4,20) completely restored the low abundant FLIPR formation.
on fibrinogen-adherent platelets up to 80.9 ± 5.6% (Figure 3D). Previous studies have indicated that dual stimulation with both CRP and thrombin results in store-independent calcium entry, involving sodium entry via TRPC channels(5,6), leading to increased PS-exposure. Store-independent calcium entry was inhibited by addition of SKF96365 together with CRP and thrombin, and resulted in decreased FLIPR formation (1.9 ± 0.5% of platelets, Figure 3E). Stimulation of store-independent calcium influx was achieved by the addition of OAG (1-oleoyl-2-acetyl-sn-glycerol), resulting in an increase in FLIPR formation (49.5 ± 5.8% of platelets, Figure 3E). This suggests that besides SOCE, also store-independent calcium entry is involved in the formation of FLIPRs.

Platelet FLIPR formation depends on cyclophilin D dependent calcium transport, and Rac1 and calpain activation.

The mitochondrial permeability transition pore (MPTP) appears an important checkpoint for shedding of platelet-derived MPs, and was furthermore shown to be an important regulator in platelet PS exposure. We therefore investigated the role of cyclophilin D, which regulates calcium transport from the mitochondria via formation of the MPTP(7,8). Inhibition of cyclophilin D by cyclosporin A resulted in a significant decrease in FLIPR formation upon addition of CRP (1.7 ± 0.8% of platelets, Figure 3F). Cyclosporin A not only inhibits cyclophilin D, but also inhibits cyclophilin A and calcineurin. Therefore, we have used direct inhibitors of cyclophilin A and calcineurin (FK506). Inhibition of cyclophilin A or calcineurin did not have any effect on FLIPR formation (Figure 3F). Furthermore, direct inhibition of MPTP opening using bongkrekic acid resulted in inhibition of FLIPR formation similar to cyclosporin A (2.4 ± 1.3% of platelets, Figure 3F). Although we could not block cyclophilin D specifically, our data indicates that FLPR formation is a cyclophilin D dependent process.

Recent studies have demonstrated that the calcium-dependent protease calpain is responsible for the formation of procoagulant platelets, which is accompanied by a closure of integrin αIIbβ3, and subsequent cleavage of cytoskeletal proteins(8,21). Our next approach was to study the role of calpain in FLIPR formation by inhibition of calpain with both calpeptin (100 μg/mL) and MDL28170 (200 μM). Inhibition of calpain completely blocked FLIPR formation, indicating that calcium-dependent calpain activation is essential for the formation of FLIPRs (Figure 3G).

Small GTPases play an essential role in the regulation of cell adhesion, motility, proliferation and differentiation. RhoA, Rac1 and CDC42 are the most characterized GTPases and known for their role in assembling and organizing the actin cytoskeleton(22,23). We have inhibited these 3 small GTPases by the addition of specific inhibitors (GSK 429286, NSC 23766 and ZCL 278, respectively). The inhibitors were added after platelet spreading together with the agonists CRP, thrombin, or a mixture of CRP and thrombin. Only Rac1 inhibition resulted in impaired FLIPR formation (Figure 3H). This small GTPase was previously shown to be essential for platelet lamellipodia formation(24).

FLIPR severing and formation of microparticles.

Reflection Interference Contrast Microscopy (RICM) was used to study the interaction of FLIPRs with the various substrates during perfusion. Spread platelets showed distinct focal membrane attachment sites to their different substrates, as visualized by the multiple dark contact areas revealed in RICM mode. The FLIPRs themselves revealed little interaction sites with the substrate (Figure 4A, DIC; Figure 4B, RICM), with the exception of the downstream tips. These observations support the concept that the membranes probably derive from the luminal aspect of the adherent cells. Interestingly, adherent platelets that formed FLIPRs showed reduced adhesive contact sites, indicating removal of membrane from the substrate. During perfusion, many FLIPRs severed from the platelets and further disintegrated into smaller fragments, presumably becoming microparticles. This severing usually occurred within seconds, but could also take several minutes, and was shear dependent.
We next captured the microparticles that were released during perfusion at the outlet of the flow chamber, i.e. in the flowthrough, for further analysis. Flowthrough derived from experiments where high numbers of FLIPRs were formed (i.e. after addition of CRP), were compared with control perfusions in the absence of CRP, and with non-perfused non-activated platelets. Membrane fractions from the flowthrough were isolated by differential centrifugation, absorbed to EM grids, and visualized by transmission EM. They consisted of elongated membrane structures of variable length and measured generally 30-70 nm in diameter (Figure 4C). They frequently exhibited a larger membrane domain at one end up to 300 nm in diameter. These extended membrane microparticles were only found in the flowthrough under conditions where FLIPRs were formed and were absent in the flowthrough of control perfusions. To further characterize these microparticles, we collected the flowthrough after perfusion with or without added CRP and performed a western blot using anti-GPIb antibodies. As expected, GPIb intensity was significantly increased in the samples with added CRP, consistent with FLIPR severing and microparticle formation (4.6 ± 0.4 fold increase in GPIb intensity, Figure 4D). Subsequent FACS analysis confirmed the presence of negatively charged phospholipids (lactadherin’), and revealed that GPIb’/lactadherin+ particles were increased in number after FLIPR formation as compared to control samples (control perfusion 1.3 ± 0.4, FLIPR perfusion 4.6 ± 1.4 fold increase in microparticles, Figure 4E).

These results indicate that conditions that promote FLIPR generation are directly coupled to the formation of procoagulant microparticles. In an attempt to visualize real-time local fibrin formation on platelet-forming FLIPRs we performed perfusions with recalcified plasma. Fibrin formation was observed throughout the whole perfusion chamber and did not allow us to conclude whether fibrin generation was selectively initiated on the procoagulant FLIPRs (data not shown).

FLIPRs attract, capture and activate circulating monocytes and neutrophils.

To study the possible role of FLIPRs in recruiting other circulating cells, we perfused isolated monocytes and neutrophils in a calcium rich buffer (1 mM CaCl₂) over FLIPR-forming platelets. Both cell types rolled over FLIPR-forming platelets, thereby decreasing their velocities (Figure 5A, supplemental movie IV). Addition of an anti-P-selectin antibody completely blocked both monocyte and neutrophil rolling on FLIPRs, indicating that this interaction is P-selectin/PSGL-1-dependent (Figure 5B, supplemental movie V). Interestingly, the temporal interactions with FLIPRs frequently resulted in severing of the FLIPR membranes, suggesting transfer of FLIPR fragments (microparticles) to the tethering cells (Figure 5A, asterisk; supplemental movie IV). To confirm that FLIPR membranes are delivered to the circulating monocytes and neutrophils, we next analyzed the presence of GPIb⁺ particles on CD14⁺ (monocytes) and CD66b⁺ (neutrophils) cells. Monocytes and neutrophils that were perfused over FLIPR forming platelets were captured and incubated with antibodies against CD14 or CD66b, and GPIb. The GPIb intensity in CD14⁺ and CD66b⁺ cells was measured by FACS and was found significantly higher in monocytes and neutrophils that were perfused over FLIPRs as compared to controls without FLIPRs (Figure 5C,D). Confocal microscopy analysis confirmed that both monocytes and neutrophils contained bound GPIb-positive platelet patches (arrowheads in Figure 5E). These patches were generally smaller than intact platelets (asterisk in Figure 5E), indicating that they represent platelet fragments presumably originating from fragmentation during the transient interactions with FLIPRs under flow.

We next measured whether monocytes and neutrophils perfused over FLIPRs become activated. An increase in CD11b expression and an increase in L-selectin shedding (as measured by a decreased CD62L intensity) are common characteristics of activated monocytes and neutrophils. Both cell types show an increased expression of CD11b after interaction with FLIPR forming platelets, as compared to perfusion in the absence of FLIPRs (Figure 5F,G). Also L-selectin shedding was increased when monocytes and neutrophils were perfused in the presence of FLIPRs as compared to perfusion in the
absence of FLIPRs (Figure 5H,I). These results show that platelet FLIPR formation contributes to the capturing of circulating monocytes and neutrophils and the simultaneous delivery of platelet membranes to the surface of these circulating cells, thereby supporting complex formation and their subsequent activation.

In vivo FLIPR formation after carotid injury.

We next asked whether such long membrane extensions are also formed in vivo. To this end we used a mouse carotid injury model. Carotid arteries were treated with FeCl₃ to induce a vascular lesion. Following 2 minutes of blood flow, samples were fixed and analyzed by SEM. We found multiple elongated membrane structures that originated from the adherent platelets, and essentially as described in our in vitro model these elongated membranes extensions are oriented down-stream the adherent platelets in the direction of the flow (arrowheads in Figure 6A,B). These data show that FLIPR formation occurs also in vivo.

DISCUSSION

In the present study we describe the formation of platelet membrane extensions, termed FLIPRs (FLow-Induced PRotusions), which develop down-stream from adherent spread platelets following activation of cyclophilin D, calpain and the small GTPase Rac1. FLIPRs derive from the luminal site of adherent platelets, and can reach lengths up to 250 μm, thereby consuming the intracellular OCS membrane stores leading to platelet disintegration and the formation of typical balloon-shaped platelets(1,2). FLIPRs severe as a result of forces generated by shear stress and transient interactions with other cells including monocytes and neutrophils, which selectively roll over FLIPRs in a P-selectin/PSGL-1 dependent fashion. These transient interactions of monocytes and neutrophils with FLIPRs resulted in the retrieval of membrane fragments from the FLIPRs, thereby forming microparticle-monocyte and microparticle-neutrophil complexes, leading to the subsequent activation of monocytes and neutrophils (Figure 7). We propose that the platelet protrusions may serve as a surface platform to capture circulating inflammatory cells. These interactions promote the selective transfer of platelet membranes to the monocytes and neutrophils that consequently become activated, leading to the progression of inflammatory processes.

Platelets store membrane in the OCS. This membrane reservoir is crucial for the platelet’s capacity to spread, but is probably equally important to form long membrane extensions(25). Loss of mean GPIb fluorescent intensity on FLIPR-forming platelets indeed indicates that OCS stores are depleted thereby supplying membrane for FLIPRs. Previous studies have shown the formation of membrane extensions on platelets interacting at high shear rates to VWF(26,27). Although these membrane extensions, termed tethers, show similarities with the present FLIPRs, they differ in several aspects. Tethers are exclusively generated on VWF at high shear rates and do not depend on platelet activation. Furthermore, tethers form upstream of platelets, and they serve to decelerate fast-moving platelets through the formation of transient adhesion points.

The formation of procoagulant balloon-shaped platelets has been described in vitro, using a combination of collagen and thrombin or alternatively through the addition of a calcium ionophore(1,2,28). Such platelet activation with strong agonists results in a sustained rise in cytosolic calcium and PS exposure, thereby supporting thrombin and fibrin generation and the formation of microparticles(1,3,28). These procoagulant platelets are referred to as balloon-shaped platelets, COAT platelets and SCIP platelets, all describing a similar phenotype(1,9,29). In our present flow model we observe essentially the same phenotype. FLIPRs are induced by a combination of physiological shear
stress and cellular activation. Upon platelet activation, intracellular calcium stores are depleted. Both store-operated and store-independent calcium entry are activated and result in a sustained rise in cytosolic calcium. MPTP, which regulate calcium transport from the mitochondria are formed under the control of cyclophilin D. Calpain is activated and responsible for the cleavage of cytoskeletal proteins. These processes together are required for FLIPR formation and high expression of PS. A limitation of our study is the lack of specific cyclophilin D inhibitors. We are therefore not able to firmly establish the role of cyclophilins in FLIPR formation. However, with no effect on FLIPR formation upon blocking of cyclophilin A or calcineurin, we believe that FLIPR formation is cyclophilin D dependent. FLIPRs bind annexin A5 and thus their formation results in an extension of the procoagulant platelet surface. Importantly, this increased platelet surface does not bind other platelets, confirming what has been described previously for SCIP platelets, due to the calcium-dependent down-regulation of the adhesive function of integrin αIIbβ3(10). Balloon-shaped platelet remnants exhibit high PS exposure on their outer membrane leaflet and supports the formation of thrombin and fibrin(1,28,29,30). While the procoagulant properties of balloon-shaped platelets are well described(28), we found no evidence that the formation of fibrin was specifically initiated by FLIPRs.

Adhesion of monocytes and neutrophils to activated endothelial cells and platelets is a key step in the inflammatory response. Our real-time flow experiments show that both circulating monocytes and neutrophils selectively roll over the long membrane extensions in a P-selectin/PSGL-1 dependent manner. In a similar fashion, neutrophil-derived membrane tethers have been shown to recruit other neutrophils, possibly through a mechanism that depends on L-selectin/PSGL-1 interaction(31). The capacity of platelets to regulate inflammatory responses is becoming firmly established. SCIP platelets have a pro-inflammatory phenotype and support the capturing and spreading of neutrophils(9). We here show that rolling of monocytes and neutrophils over FLIPRs results in transient tethering interactions and FLIPR fragmentation. Both monocytes and neutrophils were capable of capturing membrane fragments from the FLIPRs. Thus, monocyte and neutrophil-FLIPR interactions support these cells to capture platelet microparticles on their surface under flow conditions. Previous studies have shown that incubation of monocytes with procoagulant microparticles results in monocyte activation, thereby promoting their migration and differentiation(32). Additionally, monocyte adhesion to platelets resulted in the release of cytokines, thereby further promoting an inflammatory phenotype(33). Our present data, showing increased CD11b expression and L-selectin shedding on both monocytes and neutrophils after rolling over the FLIPRs, is in accordance with these studies. Furthermore, other studies have shown that platelets are able to induce a pro-inflammatory phenotype on monocytes and neutrophils upon complex formation, resulting in increased adhesive properties towards endothelial cells(34,35). This in turn is known to increase the progression of inflammatory diseases such as arthritis and atherosclerosis(11,14,36-38). Monocytes and neutrophils circulating in complex with platelets are profound markers for cardiovascular disease. Increased complexes have been measured after percutaneous coronary intervention(15), acute myocardial infarction(15), stable coronary artery disease(16), graft occlusion(39) and ischemic stroke(17). We suspect that complex formation with platelet microparticles is an underestimated phenomenon, which cannot be distinguished from complex formation with intact platelets by FACS analysis. It is therefore plausible that previous studies that claimed to measure platelet monocyte and neutrophil complexes, did in fact measure complex formation with platelet microparticles.

In an attempt to visualize the formation of these membrane extensions in vivo, we have used a mouse FeCl3 injury model to induce a vascular lesion, and visualized FLIPRs using SEM analysis. We demonstrate membrane extensions with the same characteristics as FLIPRs shown in vitro. The membrane extensions find their origin on adherent platelets and are oriented in the flow direction, defining them as FLIPRs. Whether FLIPR formation in vivo plays a role in the pro-inflammatory function of platelets remains to be shown.
In conclusion, we have described the formation of long membrane extensions that derive from adherent and activated platelets both in vitro and in vivo. FLIPRs are formed downstream the platelet along with the flow as a result of shear forces, and the formation depends on activation of cyclophilin D, calpain and small GTPase Rac1. Circulating monocytes and neutrophils interact with these membrane extensions and capture membrane fragments, thereby generating platelet microparticle-monocyte and -neutrophil complexes, and subsequent activation of monocytes and neutrophils. We propose that this mechanism of platelet membrane retrieval may be relevant for the platelet contribution in the progression of inflammatory diseases. Further studies are required to determine to what extent in vivo FLIPR formation contributes to the platelet contribution in inflammation.

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DISCLOSURES
The authors have no conflicting financial interest to declare.

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FIGURE LEGENDS

**Figure 1:** Platelet activation under shear induces a loss of platelet-membrane integrity, resulting in long membrane protrusions. (A) Washed platelets are perfused over immobilized fibrinogen (top) and fibronectin (bottom) at a flow rate of 30.3 μL/min, corresponding to a shear rate of 300 sec⁻¹. Arrowheads indicate FLIPRs formed after addition of 500 ng/mL CRP. (B) Perfusions over fibrinogen without CRP addition (top), VWF (middle) and GFOGER+CRP-XL (bottom). (C) Confocal images showing an overlay of IF staining of PF4 (red) and GPIb (green). (D) Fluorescent staining for annexin A5. (E, F) Confocal images showing IF staining of P-selectin (red) and GPIb (green). Flow direction is indicated with an arrow.

**Figure 2:** Loss of platelet integrity and cytoskeletal disassembly during FLIPR formation. (A-C) Still images from a videorecording of FLIPR formation in the direction of the flow, induced by CRP. Substrate is fibrinogen, shear rate is 300 sec⁻¹. Flow direction is indicated with an arrow. The adherent platelet cell body decreases in size (highlighted by the dashed area in A’ en C’), leaving membrane and cytoskeletal remnants as footprints. (D) Confocal images of normal (left panel) and FLIPR-forming platelets (right panel). Platelets were immunostained with anti-GPIb and actin as indicated. Disassembly of actin in cell body and presence of actin nodules in the FLIPRs (arrowheads in D). (E) Quantification of the mean fluorescent intensity of normal and FLIPR-forming platelets. 20 platelets from 5 different donors were measured. Error bars indicate standard deviation. (F) Immunostaining of α-tubulin (red: GPIb; green: α-tubulin [bottom]). Note the disorganized staining of FLIPR forming platelet. Normal platelets indicated on the left, platelets with FLIPR on the right. Arrowheads indicate disrupted cytoskeleton. (G, H) Whole mount TEM images of platelets and FLIPRs on electron microscopy formvar grids. Arrowheads indicate thickened membrane blebs on the FLIPR. Scale bars represent 200 nm.

**Figure 3:** Cyclophilin D dependent Ca²⁺ signaling and activation of Rac1 and calpain leads to FLIPR formation. (A) Perfusion of citrated platelet rich plasma in absence and presence of calcium chelator EGTA (5 mM). (B) Calcium flux indicated by an increase in Oregon green signal during perfusion over fibrinogen after CRP activation. Arrow indicates the moment of FLIPR formation. (C) Perfusion of washed platelets over fibrinogen in the presence and absence of calcium (1 mM), CRP (500 ng/mL) and/or thrombin (IIa, 0.5 U/mL). (D) Perfusion over fibrinogen followed by addition of CRP with or without thapsigargin (1 μM) or 2-APB (50 μM). (E) Perfusion over fibrinogen followed by the addition of CRP and thrombin in the presence and absence of SKF96365 (100 μM) and washed platelets alone in the presence and absence of OAG (50 μg/mL). (F) Washed platelets were perfused over fibrinogen followed by the addition of CRP with or without cyclosporin A (CsA, 4 μM), cyclophilin A inhibitor (CypA inhibitor, 1μg/mL) calcineurin inhibitor (CN inhibitor, 50 μM) and bongkrekic acid (BA, 50 μM). (G) Calpeptin (100 μg/mL) and MDL 28170 (200 μM) were added with CRP to washed platelets perfused over fibrinogen. Results of three independent flow experiments were quantified. Error bars indicate standard deviation. (H) Perfusion over fibrinogen followed by addition of CRP with or without GSK 429286 (RhoA inhibitor, 1 μM), NSC 23766 (Rac1 inhibitor, 300 μM), and ZCL 278 (CDC42 inhibitor, 50 μM).

**Figure 4:** FLIPR formation leads to the formation of platelet microparticles. (A,B) DIC recording (A) and corresponding RICM picture (B) showing surface contact in black and no surface contact in white. Arrowheads indicate transient interaction of FLIPRs with the substrate. Flow direction is indicated with an arrow. (C) Whole-mount TEM images of the flowthrough captured after perfusion. Elongated membrane fragments of ≥ 1.5 μm long and ~100 nm thick. (D) Western blot analysis of GPIb on microparticle fractions isolated from the flowthrough. Data are depicted as mean GPIb intensity relative to the fractions obtained from control without perfusion. (E) FACS analysis on microparticle-rich flowthrough. The number of GPIb⁺/lactadherin⁺ particles was quantified. Data are expressed as fold...
increase in the amount of microparticles compared to control without perfusion. Averages are shown of three independent flow experiments. Error bars indicate standard deviation.

**Figure 5:** FLIPRs capture monocytes and neutrophils from the circulation, leading to activation and complex formation with platelet microparticles. (A) Monocytes and neutrophils were perfused over FLIPRs to study rolling. Monocyte perfusion depicted in these images, rolling cells are indicated with an arrowhead. (B) Anti-P-selectin addition resulted in complete inhibition of monocyte and neutrophil rolling. Flow direction is indicated with an arrow. (C, D) Monocytes (C) and neutrophils (D) were captured in the flowthrough and the GPIb intensity on CD14+ and CD66b+ cells was measured using FACS analysis. (E) Captured monocytes from the flowthrough were stained for CD14 and GPIb. Intact platelet is indicated with an asterisk. Size of the adhered GPIb+ particles indicates the adhesion of platelet microparticles. (E) Captured monocytes and neutrophils in flowthrough were analyzed for activation markers CD11b (F: monocytes, G: neutrophils) and L-selectin shedding (by a decrease in CD62L intensity (H: monocytes, I: neutrophils). Results of three independent experiments were quantified. Error bars indicate standard deviation.

**Figure 6:** FLIPR formation in an in vivo mouse artery injury model. A-B) SEM images of FeCl3 injured carotid artery 2 min after application of the injury. Long membrane protrusions (arrowheads) originating from adherent platelets (p) are formed in the direction of the flow. Drop-shaped red cells morphology (star in B) are indicative for the flow direction. Magnification A, 6500x; B, 3500x.

**Figure 7:** Model of FLIPR formation and membrane transfer. Platelet adhesion and spreading on a physiological surface. Extracellular calcium entry and depletion of intracellular stores (DTS: dense tubular system, mito: mitochondria) raise the cytosolic calcium concentration. High cytosolic calcium and shear forces induce the formation and elongation of long membrane protrusions (FLIPRs). Phosphatidylserine (PS) and P-selectin are expressed on the platelet and FLIPR membrane. Monocytes and neutrophils roll over FLIPRs, retrieve membrane fragments, and become activated.
Novelty and Significance

What Is Known?

- Platelets are important in both hemostasis and inflammation.
- Activated platelets have procoagulant activity and generate microparticles.
- Activated platelets are furthermore able to bind leukocytes, thereby promoting inflammatory responses.

What New Information Does This Article Contribute?

- Activated platelets under flow generate extremely long membrane protrusions.
- These protrusions can severe due to shear, resulting in the formation of platelet microparticles.
- Leukocytes are able to bind to the long membrane strands resulting in leukocyte activation.

The dynamic process leading to procoagulant and pro-inflammatory platelet formation has never been visualized. We therefore aimed to visualize this dynamic process under physiological flow. Exposing platelets to shear and agonists results in the formation of Flow-Induced PRotrusions, FLIPRs, both in vitro as well as in a mouse carotid injury model in vivo. Severing of FLIPRs from adhered platelets results in microparticle formation and the formation of balloon-shaped platelets. Balloon-shaped platelets were demonstrated before and are known to exhibit procoagulant and pro-inflammatory properties. Previous studies furthermore show the importance of mitochondrial calcium regulation for the generation of procoagulant activity, and the requirement for the calcium-dependent protease calpain in the generation of microparticles. We show that both are also required for FLIPR formation. In addition, monocytes and neutrophils are able to role over the FLIPRs and retrieve the membrane fragments as microparticles on their surface. This results in activation of monocytes and neutrophils, promoting the inflammatory response. FLIPRs formation as presented in our current study, provides a mechanistic model for platelet membrane transfer and the generation of monocyte/neutrophil-microparticle complexes. We propose that the formation of FLIPRs in vivo contributes to the well-established pro-inflammatory function of platelets and platelet-derived microparticles.
Figure 1
Figure 3
Figure 4
Figure 7

Adhesion

Spreading

FLIPR formation

FLIPR elongation and platelet disintegration

Transition to pro-inflammatory phenotype

Transfer of membrane and monocyte/neutrophil activation
Flow-Induced PRotusions (FLIPRs): A Platelet-Derived Platform for the Retrieval of Microparticles by Monocytes and Neutrophils
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Flow-Induced PRotusions (FLIPRs): a platelet-derived platform for retrieval of microparticles by monocytes and neutrophils

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Detailed Materials and Methods

Materials

Human serum albumin (HSA) was purchased from MP Biomedicals (ImmunoOne fraction V; Amsterdam, the Netherlands). Fibrinogen was obtained from Enzyme Research Laboratories (Swansea, UK) and von Willebrand Factor (VWF) from Biotest AG (Dreieich, Germany). GFOGER and CRP-XL were produced as described before (1,2). RGD-W was purchased from the NKI (Amsterdam, the Netherlands). Calpeptin, 2-aminoethoxydiphenylborate (2-APB) and bongkrekic acid were obtained from Enzo Life Sciences (Antwerpen, Belgium), and MDL 28170, thapsigargin and FK-506 from Sigma (Zwijndrecht, the Netherlands). Cyclophilin A inhibitor was purchased from EMD Millipore (Billerica, USA) and 1-oleoyl-2-acetyl-glycerol (OAG) from Santa Cruz (Heidelberg, Germany).

The following antibodies were used for immunofluorescent staining: anti-GPIb (HIP-1) (eBiosciences, Vienna, Austria); anti-P-selectin, anti-fibrinogen, and anti-GPIb (SZ2) (Santa Cruz); anti-PF4 and anti-βTG (R&D systems); annexin A5-FITC (Biovision, MountainView, CA, USA); phalloidin-FITC (Invitrogen, Bleiswijk, the Netherlands); and anti-α-tubulin (eBiosciences). Secondary antibodies labeled with Alexa fluor 488, Alexa fluor 555, and Alexa fluor 680 were purchased from Invitrogen. Lactadherin-FITC (Haematologic Technologies, Essex Junction, VT, USA) and FITC-, PE- or APC-labeled monoclonal antibodies against GPIb, P-selectin, CD14, CD66b, CD11b and CD62L (BD Biosciences, Franklin Lakes, NJ, USA) were used for FACS analysis.

Blood collection

Blood was collected from healthy volunteers who had not been on anticoagulant or antiplatelet medication for at least 10 days before blood withdrawal. The study was approved by the University Medical Center Utrecht (UMCU) Ethics Committee. Blood was anticoagulated with 10% sodium citrate (3.2% w.v.). For annexin A5 adhesion stainings, blood was anticoagulated using 100 U/mL hirudin. Platelet rich plasma (PRP) and washed platelets were prepared as described before (3). Platelet count was determined using CellDyn1800 (Abbott, Hoofddorp, the Netherlands) and set to 350x 10^9/L.

Real-time perfusion studies

Glass coverslips were cleaned with chromic-sulfuric acid (Sigma), rinsed with dH2O and placed in a polydimethylsiloxane (PDMS) perfusion chamber with a channel height of 75 µm and width of 2 mm. The channels were coated with fibrinogen (100 µg/mL), VWF (10 µg/mL), fibronectin (100 µg/mL) or GFOGER+CRP-XL (100 µg/mL and 200 µg/mL, respectively) for 1.5 hours at room temperature and blocked with 1% HSA overnight at 4°C. PRP and washed platelets were perfused using a syringe pump (Harvard Apparatus, Holliston, MA, USA) at a shear rate of 300 sec⁻¹. Washed platelets that were perfused over fibrinogen were activated using collagen related peptide (CRP, 500 ng/mL) after 15 minutes of perfusion. FLIPR formation was visualized with an inverted microscope (Zeiss observer Z.1, Carl Zeiss, Sliedrecht, the Netherlands). Movies and snapshots were recorded with differential interference contrast (DIC) microscopy, using a 40x/1.25 oil or 100x/1.25 oil EC-plan Neofluor objective (Carl Zeiss). For reflection interference contrast microscopy (RICM) analysis, a 63x/1.25 oil EC-plan Neofluor antifluor objective (Carl Zeiss) was used in combination with an HBO lamp. All images were analyzed with AxioVision software (Release 4.6, Carl Zeiss) and ImageJ software (Release 1.41, National Institutes of Health, Bethesda, MD, USA).

Immunofluorescent staining of adhered platelets and FLIPRs

After perfusion, adhered platelets and FLIPRs were fixed under flow using 2% paraformaldehyde (PFA). Samples were blocked using 10% normal goat serum for 30 minutes. Immunofluorescent staining was performed using antibodies against GPIb, P-selectin, PF4, β-TG, fibrinogen, α-tubulin and CD14. Secondary antibodies containing either AF488 or AF555 were used.
to visualize the stainings. Stains were analyzed using a Zeiss LSM 510 meta confocal microscope (Carl Zeiss).

**Determination of GPIb distribution across membranes**

Images of GPIb-stained platelets were taken using confocal microscopy. ImageJ was used to determine the intensity of the staining, with the histogram option being directed at selected platelets with and without a FLIPR. The intensity of the normal platelets was set at 100% and used to calculate the intensity of the platelets with a FLIPR in the same microscopic picture.

**Real-time calcium flux microscopy**

Calcium flux was visualized in washed platelets (suspended in HEPES-Tyrode buffer, pH 7.3) that had been incubated with 1 µM Oregon green BAPTA-1, AM (Invitrogen) at 37°C for 30 minutes. Platelets were centrifuged again in the presence of 10ng/mL PGI2 and resuspended in HEPES-Tyrode buffer (pH 7.3) with 1 mM CaCl2. Perfusion was performed and calcium flux was visualized and analyzed using AxioVision software (Release 4.6, Carl Zeiss).

**FACS analysis and western blot on microparticles**

Washed platelets were perfused in a calcium rich buffer over fibrinogen. FLIPR formation was created by perfusion of 500 ng/mL CRP after 15 minutes (=perfusion with FLIPRs). The control perfusion was not activated with CRP (=perfusion no FLIPRs). Flowthroughs were collected and centrifuged twice at 1500xg for 15 minutes in the presence of PGI2 and 20 mM EDTA to prevent platelet activation during centrifugation in the presence of CRP remnants. For FACS analysis, the supernatant containing microparticles was then incubated with antibodies against GPIb and lactadherin for 20 minutes and fixed using 0.2% formaldehyde in 0.9% NaCl. All samples were analyzed using the FACSCanto from BD Biosciences. Microparticles were gated based on forward- and side-scatter properties, and the number of GPIb+/lactadherin+ events was counted and compared to that of the isotype controls. For westernblots, the supernatant was centrifuged at 20,000xg for 1 hour, and the microparticle-containing pellet resuspended in sample buffer in the presence of dithiothreitol (DTT). Proteins were separated by SDS-PAGE and stained with anti-GPIb (SZ-2).

**Electron microscopy of FLIPRs and microparticles**

Platelets were perfused over fibrinogen-coated formvar grids, and fixed with a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer. Platelets and FLIPRs were counterstained with uranyl acetate and analyzed in a JEOL 1200CX electron microscope at 80 kV (JEOL, Nieuw-Vennep, The Netherlands). Microparticle-containing samples were collected after perfusion (as described in the FACS and western blot section) at the outlet of the perfusion chamber and isolated using differential centrifugation. Isolated vesicle fractions were examined in the electron microscope after uranyl staining and embedding.

**Monocyte and neutrophil perfusions**

For monocyte isolation, citrated whole blood was diluted with HEPES-Tyrode buffer (pH 7.3) and a Ficoll separation was performed. The pellet was resuspended in HEPES-Tyrode buffer (pH 7.3) with 1 mM CaCl2. Monocytes were isolated using CD14+ magnetic beads (Miltenyi Biotec, Leiden, the Netherlands). Neutrophils were isolated from the supernatant obtained after static incubation of blood (from which platelets were already isolated) with a 6% Dextran/0.9% NaCl solution. The supernatant was spun down and the pellet was shocked with ice-cold water. 0.6M KCl was added, followed by HBS and cells were again spun down. Neutrophils were isolated using a Ficoll separation and resuspended in HEPES-Tyrode buffer (pH 7.3) with 1 mM CaCl2. The purity of both monocytes and neutrophils was established using the CellDyn1800, which measures the cell type based on light scattering, as well as with FACS analysis for CD14 and CD66b. Both types of analysis indicated a purity of >98%.

Washed platelets were perfused for 15 minutes followed by activation with CRP for 5 minutes. Monocytes and neutrophils were then perfused, and rolling and adhesion were studied during real-time perfusion. The flowthrough during these perfusions were captured and stained with CD14 (monocytes), CD66b (neutrophils), GPIb, CD11b or CD62L for 20 minutes and fixed using 0.2%
formaldehyde in 0.9% NaCl. CD14$^+$ or CD66b$^+$ cells were gated and GPIb mean fluorescence intensity was measured within this gate to analyze the presence of platelet (microparticles) on the monocytes or neutrophils. All samples were analyzed using the FACSCanto from BD Biosciences.

**In vivo FLIPR formation in a mouse carotid injury model**

All procedures for animal experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals as defined by European laws. Mice (8-10 weeks) were anesthetized by intra peritoneal administration of ketamine (100 mg kg$^{-1}$) and xylazine (20 mg kg$^{-1}$). The common carotid arteries were exposed and vascular injury was induced by application of 7.5% FeCl$_3$ saturated Whatmann filter paper on top of the carotid artery for 2 minutes. The arteries were fixed with 2.5% glutaraldehyde by transcardiac perfusion. The injured artery was excised and post-fixed in 2.5% glutaraldehyde overnight. The isolated segments were cut open in the middle and dehydrated in increasing concentrations of ethanol, followed by treatment with hexamethyldisilazane. Samples were glued on a coverslip with the lumen oriented upwards. The coverslips were sputter-coated with gold and analyzed at 5 kV using a FEG Sirion SEM (FEI, Hillsboro, USA).

**Statistics**

A Mann-Whitney U test was performed to compare the difference between 2 conditions. A p-value of less than 0.05 was considered significant. All data analyses were performed with computer software (SPSS-.pc Version 15.0, SPSS Inc., Chicago, IL, USA).
Online supplemental data

Supplemental movie I: FLIPR formation after stimulation with CRP. Platelet adhesion and spreading on fibrinogen was observed during the first 10 minutes of perfusion. After perfusion with CRP, adhered platelets start forming FLIPRs in the direction of the flow. Note that the platelet body decreases in size as the FLIPR elongates. Video was recorded at four frames/second using DIC microscopy, and actual speed is displayed. Flow direction is indicated with an arrow.

Supplemental movie II: Overview of FLIPR formation. CRP was added to platelets spread on fibrinogen substrate. Multiple platelets are shown forming long membrane protrusions. The video was recorded at four frames/second using DIC microscopy. Actual speed is displayed. Flow direction is indicated with an arrow.

Supplemental movie III: FLIPR elongation and platelet disintegration. Real-time video recording of a spread platelet adhered to fibrinogen. Extension of the membrane is followed real time to show the length of the FLIPR. Note that at the end of the recording, the morphology of the spread platelet is changed and the platelet starts to disintegrate. Video was recorded at four frames/second using DIC microscopy, and actual speed is displayed. Flow direction is indicated with an arrow.

Supplemental movie IV: Monocyte rolling over FLIPRs. FLIPRs were created by perfusing washed platelets over fibrinogen, followed by CRP activation and isolated CD14⁺ monocytes were perfused subsequently. Note the FLIPR membrane that is disappearing due to retrieval of membrane by the rolling monocytes. Video was recorded at four frames per second using DIC microscopy and actual speed is displayed. Flow direction is indicated with an arrow.

Supplemental movie V: Inhibited monocyte rolling by blocking P-selectin. FLIPRs were created by perfusing washed platelets over fibrinogen, followed by CRP activation and isolated CD14⁺ monocytes were perfused subsequently in the presence of anti-P-selectin. Video was recorded by DIC microscopy at a speed of four frames per second. Data were collected and the video is displayed at actual speed. Flow direction is indicated with an arrow.
References


