Circulation Research

ORIGINAL RESEARCH

Rivaroxaban Reduces Arterial Thrombosis by Inhibition of FXa-Driven Platelet Activation via Protease Activated Receptor-1

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RATIONALE: A reduced rate of myocardial infarction has been reported in patients with atrial fibrillation treated with FXa (factor Xa) inhibitors including rivaroxaban compared with vitamin K antagonists. At the same time, low-dose rivaroxaban has been shown to reduce mortality and atherothrombotic events in patients with coronary artery disease. Yet, the mechanisms underlying this reduction remain unknown.

OBJECTIVE: In this study, we hypothesized that rivaroxaban's antithrombotic potential is linked to a hitherto unknown rivaroxaban effect that impacts on platelet reactivity and arterial thrombosis.

METHODS AND RESULTS: In this study, we identified FXa as potent, direct agonist of the PAR-1 (protease-activated receptor 1), leading to platelet activation and thrombus formation, which can be inhibited by rivaroxaban. We found that rivaroxaban reduced arterial thrombus stability in a mouse model of arterial thrombosis using intravital microscopy. For in vitro studies, atrial fibrillation patients on permanent rivaroxaban treatment for stroke prevention, respective controls, and patients with new-onset atrial fibrillation before and after first intake of rivaroxaban (time series analysis) were recruited. Platelet aggregation responses, as well as thrombus formation under arterial flow conditions on collagen and atherosclerotic plaque material, were attenuated by rivaroxaban. We show that rivaroxaban's antiplatelet effect is plasma dependent but independent of thrombin and rivaroxaban's anticoagulatory capacity.

CONCLUSIONS: Here, we identified FXa as potent platelet agonist that acts through PAR-1. Therefore, rivaroxaban exerts an antiplatelet effect that together with its well-known potent anticoagulatory capacity might lead to reduced frequency of atherothrombotic events and improved outcome in patients.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: anticoagulants ■ myocardial infarction ■ platelet aggregation ■ rivaroxaban ■ thrombosis

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Nonvitamin K anticoagulants are already first choice in stroke prevention of patients with nonvalvular atrial fibrillation (AF).[1] Currently, 2 pharmacologically different groups of nonvitamin K anticoagulants are used: FXa (factor Xa) and FIIa (factor IIa) inhibitors.[2] Compared to vitamin K antagonists, an improved benefit/risk ratio regarding the prevention of embolic events and bleeding has been shown for both types of nonvitamin K

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Novelty and Significance

What Is Known?

- Rivaroxaban treatment is associated with reduced incidence of ischemic events.

What New Information Does This Article Contribute?

- FXa (factor Xa) acts as potent platelet agonist through activation of PAR (protease-activated receptor)-1, driving arterial thrombosis.
- Rivaroxaban reduces platelet activation and thrombus formation.

Rivaroxaban treatment is associated with reduced incidence of ischemic events. We identified FXa as potent platelet agonist. FXa directly induces platelet activation and thrombus formation on atherosclerotic plaque material, independently of thrombin. Rivaroxaban treatment abolished FXa-mediated platelet effects in a dose-dependent manner. Our study identified an unknown antiplatelet effect of Rivaroxaban. Together with its anticoagulative potential, this might contribute to reduced ischemic events.

Nonstandard Abbreviations and Acronyms

AF	atrial fibrillation
FIIa	factor IIa
FXa	factor Xa
GP	glycoprotein
ICAM	intercellular adhesion molecule
MEA	multiple-electrode impedance aggregometry
MI	myocardial infarction
MMP	metalloproteinase
PAC	procaspase activating compound
PAR-1	protease-activated receptor 1
PI3K	phosphoinositide 3-kinase
PLC	phospholipase C
PRP	platelet-rich plasma
ROCK	rho-associated, coiled-coil containing protein kinase
TIMI	Thrombolysis In Myocardial Infarction
TRAP	thrombin receptor-activating peptide
WP	washed platelets

anticoagulants.[2–5] A recent meta-analysis that included 9 large randomized clinical trials, testing the FXa inhibitor rivaroxaban in different clinical indications, revealed a reduced risk for myocardial infarction (MI)[6] in patients receiving rivaroxaban compared with different comparators. This overall effect on MI was primarily driven by results from the rocket-AF and Atlas acute coronary syndrome 2-TIMI (Thrombolysis In Myocardial Infarction) 51 trials that both investigated patient populations at high risk for ischemic coronary events. Rocket-AF compared rivaroxaban against warfarin for stroke prevention in patients with AF and found a lower number of MI and a 14% decrease of ischemic cardiac events under rivaroxaban.[7] Atlas acute coronary syndrome 2-TIMI 51[8] compared low-dose rivaroxaban (2.5 mg twice daily) versus placebo treatment on top of standard antiplatelet therapy in patients with recent acute coronary syndrome and found a reduced risk for MI. Furthermore, the recent randomized COMPASS study (Cardiovascular Outcomes for People Using Anticoagulation Strategies)[9] observed an improved survival in patients with stable coronary artery disease in patients receiving rivaroxaban on top of aspirin compared to aspirin monotherapy. This suggests that rivaroxaban reduces ischemic events in patients with advanced atherosclerosis.

At sites of atherosclerotic plaque rupture, exposure of highly thrombogenic matrix proteins and procoagulant factors triggers platelet recruitment, which is accompanied by the activation of the extrinsic and intrinsic coagulation cascades that merge into the formation of the prothrombinase complex. During the early phase of arterial thrombosis, FXa is generated on procoagulant platelets and the injured endothelium surrounding the lesion site.[10,11] The major outcome of FXa generation is thrombin and subsequent fibrin formation. Hence, rivaroxaban is considered to act primarily by inhibiting coagulation through prevention of FXa-dependent thrombin generation.

However, against the background of reduced atherothrombotic events in recent clinical trials, we hypothesized that in addition to its anticoagulant effect, rivaroxaban might exert a hitherto unknown antiplatelet effect that in concert with its well-known anticoagulatory capacity contributes to the reduced frequency of coronary ischemic events. In this study, we found that FXa acts as potent platelet agonist independently of thrombin, triggering platelet activation through PAR-1 (protease-activated receptor 1) which is inhibited in the presence of rivaroxaban. Furthermore, rivaroxaban dose-dependently reduced platelet activation, aggregation and thrombus formation under flow conditions and in vivo.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Antiplatelet effects were measured in patients before and after rivaroxaban medication as well as a cross-sectional analysis. Light-transmittance aggregometry and multiplate impedance aggregometry (MEA), flow chamber experiments, flow cytometry, and enzyme-linked immunosorbent assay and FXa activity assays were conducted to assess platelet response. In vivo thrombus formation in mice and histological analyses were conducted to measure rivaroxaban's effects on thrombus formation. A detailed description of all methods is presented in the Online Data Supplement.

RESULTS

Rivaroxaban Attenuates Arterial Thrombus Formation In Vivo

We first investigated the effect of rivaroxaban on thrombus formation visualized by in vivo microscopy in a mouse model of arterial thrombosis after inducing a Fe-(III) chloride injury of the common carotid artery. Treatment of C57BL/6J wild-type mice with a single dose of rivaroxaban (6 mg/kg body weight) resulted in clinically relevant plasma levels as known in patients (Figure 1A). While rivaroxaban did not affect the time to thrombotic occlusion of the artery (Figure 1B), we found less stable thrombi with an increased frequency of thrombus fragment embolization compared with vehicle treatment within the observation period of 60 minutes (Figure 1C, Online Movie I). Importantly, we observed a similar phenotype after aspirin treatment in an earlier study.[12] Of note, vitamin K antagonist treatment for 48 hours before injury lead to thrombus protection, underscoring its potent antithrombotic effect.[13] Histological analysis of thrombus confirmed a less compact thrombus structure with reduced platelet and fibrinogen deposition under rivaroxaban (Figure 1D through 1F; isotype control stainings are shown in Online Figure IA). Because platelets are important players in thrombus stability,[14] our findings can be interpreted in the way that rivaroxaban exerts antiplatelet effects.

Rivaroxaban Reduces Platelet Aggregation

To further dissect rivaroxaban's potential antiplatelet from its anticoagulatory effect, we next addressed the impact of rivaroxaban on human platelet aggregation using blood obtained from patients under rivaroxaban treatment. Hence, patients under chronic rivaroxaban medication or before and after initiation of rivaroxaban were recruited. Baseline characteristics are shown in Online Table I.

To exclude potential thrombin mediated effects, we studied whole blood aggregation using MEA in hirudin-anticoagulated blood from rivaroxaban patients and patients without anticoagulants upon stimulation with various agonists, including pathophysiological relevant human atherosclerotic plaque. As shown in Figure 2A, we found reduced aggregation responses for all tested stimuli. Earlier studies suggested that platelet reactivity was increased in patients with AF compared to subjects with sinus rhythm,[15] which could represent a confounding factor. To exclude this and other potential confounding factors, including sex-specific differences and comorbidities, such as coronary artery disease, we used a second approach and analyzed platelet responses over time. In brief, we performed MEA in hirudin-anticoagulated blood from the same patient before and 4 hours after receiving a single dose of rivaroxaban (20 mg). Again, platelet aggregation was attenuated after rivaroxaban treatment (Figure 2B).

As MEA measurements were performed in whole blood, they could be influenced by ADP release from erythrocytes.[16] Hence, we next analyzed platelet aggregation in citrate-anticoagulated platelet-rich plasma (PRP). Again, we excluded a thrombin dependency of our experimental setup, as aggregation responses were not altered in the presence of thrombin inhibitors hirudin and bivalirudin, respectively (Online Figure IB).

As shown in Figure 2C, a single dose of rivaroxaban attenuated platelet aggregation following stimulation with ADP, collagen, and TRAP (thrombin receptor-activating peptide). These differences were not due to an altered receptor surface expression, as determined by flow cytometry for collagen receptor GP (glycoprotein)-VI, ADP receptors P2Y12 and P2Y1 and platelet expressed PAR receptor family members (Online Figure IC). To evaluate whether rivaroxaban's antiplatelet effects are directly platelet or plasma dependent, we conducted a plasma-switch experiment using hirudin-anticoagulated blood (Figure 2D) as shown in the incubation scheme (Online Figure II). We found that the addition of rivaroxaban naïve plasma (t:0h) to rivaroxaban exposed (t:4h) washed platelets (WP) could rescue aggregation responses, yielding a similar aggregation response as found in the absence of rivaroxaban. We further elaborated on this finding and found that rivaroxaban affects collagen-induced aggregation responses only in PRP but not in WP (Figure 2E). Together these data show that rivaroxaban antiplatelet effects are plasma dependent.

Next, we asked whether MEA aggregation responses were dependent on rivaroxaban plasma levels. We found that higher plasma levels were associated with a reduced aggregation response (Figure 2F), indicating that rivaroxaban's antiplatelet effects were dose-dependent. To define whether the antiplatelet effects were drug-specific for rivaroxaban or represent a class effect of FXa inhibitors, we repeated the experiments in patients receiving apixaban, another clinically approved FXa inhibitor. Interestingly, we found the same inhibitory effect on platelet aggregation in response to apixaban, suggesting a group rather than a substance-specific effect (Figure 2G).

Rivaroxaban Reduces Platelet Adhesion and Thrombus Formation Under Flow Conditions

To study arterial thrombus formation in patient blood under more physiological conditions, we performed

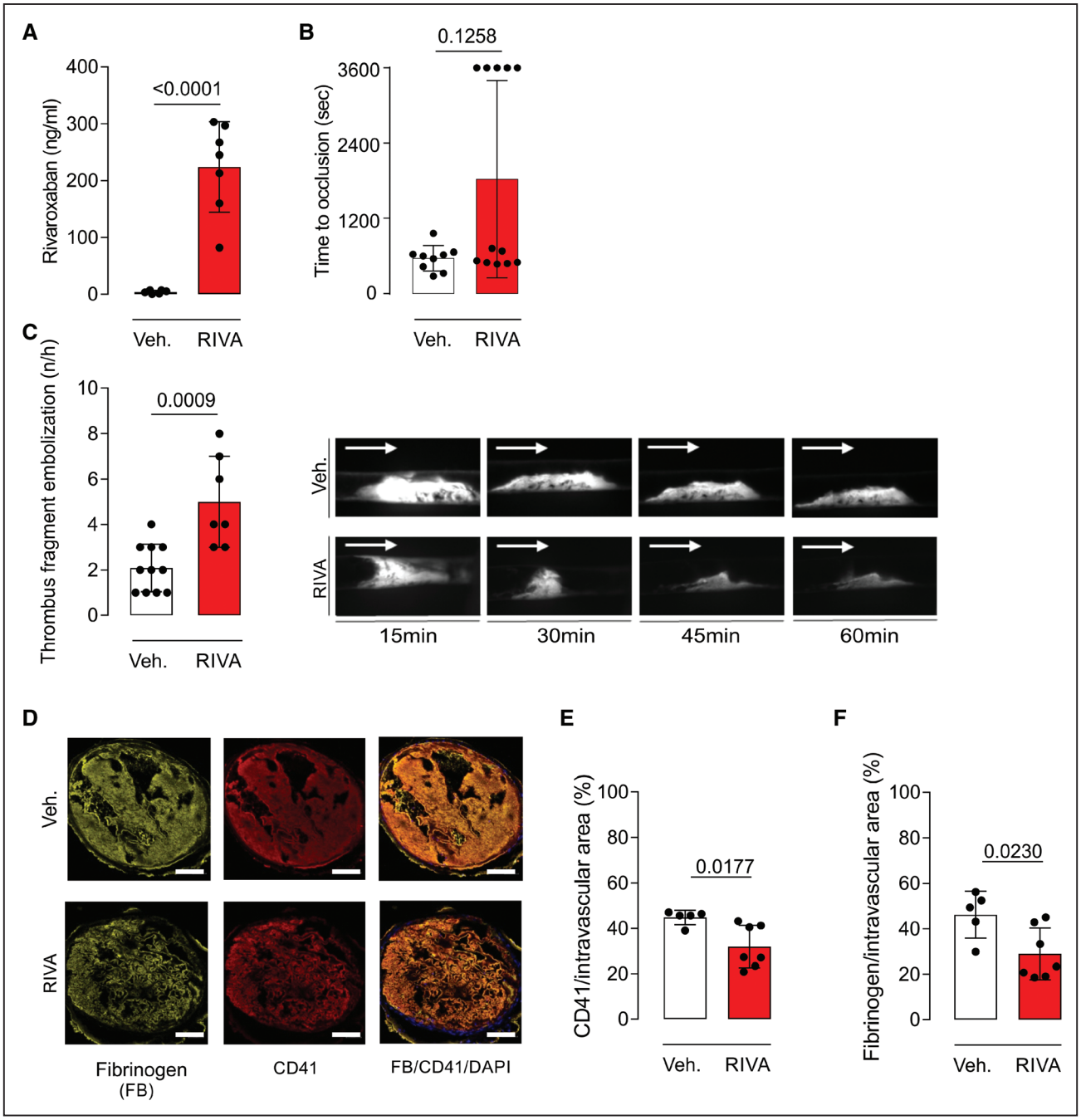

Figure 1. Rivaroxaban (RIVA) attenuates thrombus stability in vivo.
A, Wild-type (WT) mice received a single dose of RIVA (6 mg/kg body weight) or vehicle. RIVA plasma concentrations were determined 60 min after application (vehicle n=6 vs RIVA n=7, unpaired *t* test, $P<0.0001$, Shapiro-Wilks test [SW] showed normal distribution). Treated animals underwent a Fe-(III) chloride carotid artery injury. Thrombus formation was visualized over 60 min using fluorescence microscopy. Time to first occlusion (**B**; vehicle n=9 vs RIVA n=12, Mann-Whitney test, $P=0.1258$, SW showed no normal distribution) and thrombus fragment embolization (n/h) (**C**; vehicle n=11 vs RIVA n=7, unpaired *t* test, $P=0.0009$, SW showed normal distribution) were recorded. Representative fluorescent images of RIVA or vehicle treated animals 15, 30, 45, and 60 min after induction of injury are shown. Arrows indicate the direction of flow and represent 500 µm. **D**–**F**, Arterial thrombus composition of explanted thrombi form RIVA and vehicle treated animals were analyzed using confocal microscopy. Representative immunofluorescence images are shown (**D**). Intravascular surface coverage of platelet (CD41) (**E**; vehicle n=5 vs RIVA n=7, Mann-Whitney test, $P=0.0177$, SW showed no normal distribution) and fibrinogen (**F**; vehicle n=5 vs RIVA n=7, unpaired *t* test, $P=0.0230$, SW showed normal distribution) was quantified. Data shows individual animals' means±SD; scale bar represents 100 µm. All data show means±SD.

flow chamber assays. The early phase (up to 3 minutes) of platelet adhesion and aggregate formation was investigated under laminar flow conditions with arterial shear (10 dyn/cm^2) on collagen. Using fluorescent microscopy, we observed reduced platelet adhesion and thrombus formation after 3 minutes of flow in

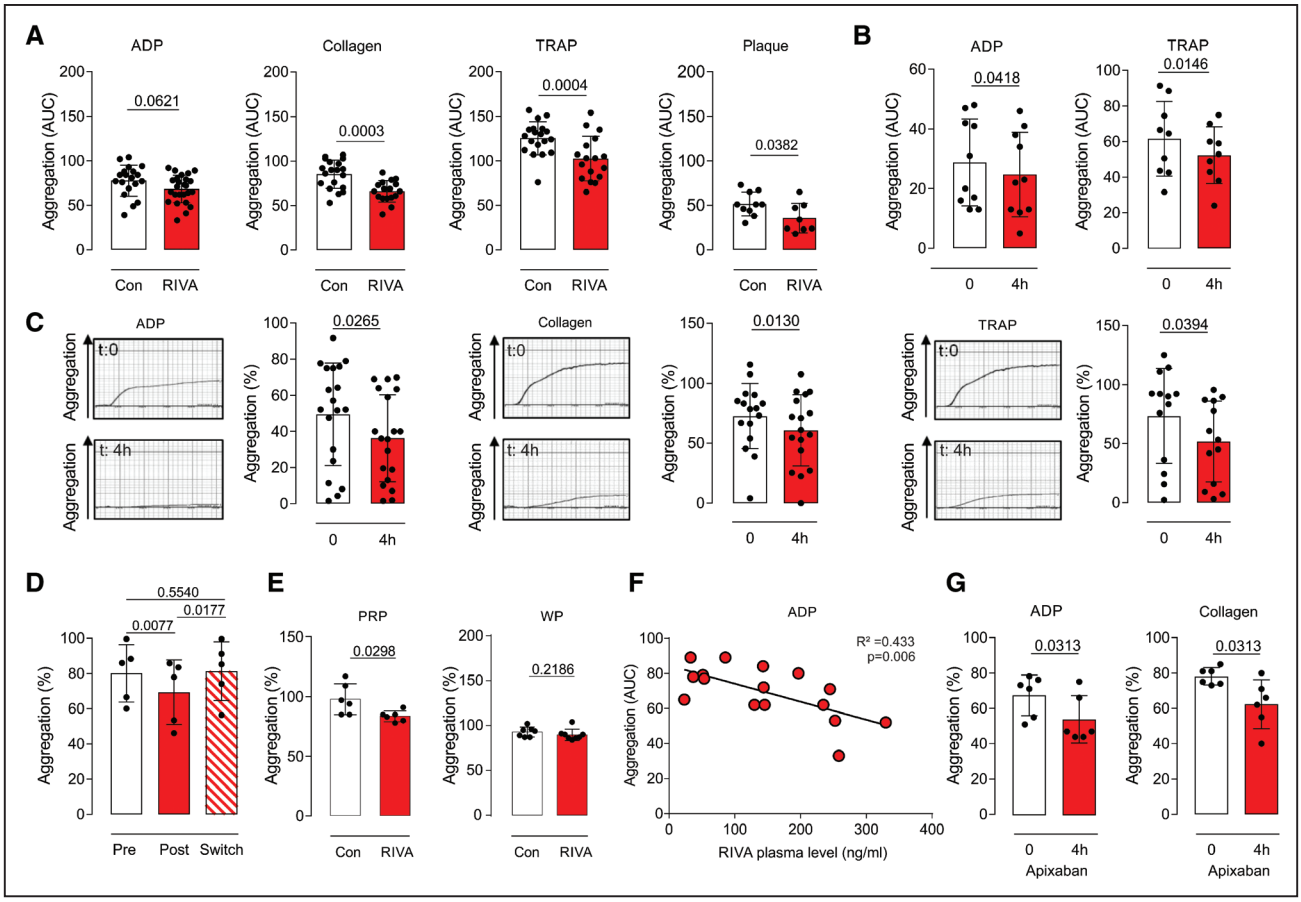

Figure 2. Platelet aggregation is reduced under rivaroxaban (RIVA).
A, Whole blood multiple-electrode aggregometry (MEA) upon stimulation with 5 µmol/L ADP, 2 µg collagen, 12 µmol/L TRAP (thrombin receptor-activating peptide) and human atherosclerotic plaque material (plaque) within the cross-sectional cohort (ADP–control n=19 vs RIVA n=25, unpaired *t* test, *P*=0.0621, Shapiro-Wilks [SW] showed normal distribution; collagen–control n=19 vs RIVA n=17 unpaired *t* test, *P*=0.0003, SW showed normal distribution; TRAP–control n=19 vs RIVA n=17, unpaired *t* test, *P*=0.0004, SW showed normal distribution; plaque–control n=10 vs RIVA n=8, unpaired *t* test, *P*=0.0382, SW showed normal distribution) and (**B**) upon ADP and TRAP for the time series analysis was quantified (ADP–before rivaroxaban n=10 vs after rivaroxaban n=10, paired *t* test, *P*=0.0418, SW showed normal distribution; TRAP–before rivaroxaban n=9 vs after rivaroxaban n=9, paired *t* test, *P*=0.0146, SW showed normal distribution). **C**, Light transmission aggregometry (LTA) in platelet-rich plasma (PRP) was performed within the time series analysis for the indicated stimuli (ADP–before rivaroxaban n=19 vs after rivaroxaban n=19, paired *t* test, *P*=0.0265, SW showed normal distribution; collagen–before rivaroxaban n=17 vs after rivaroxaban n=17, paired *t* test, *P*=0.0130, SW showed normal distribution; TRAP–before rivaroxaban n=13 vs after rivaroxaban n=13, paired *t* test, *P*=0.0394, SW showed normal distribution). **D**, Plasma-switch experiments were performed as shown in the incubation scheme (Online Figure II). Resuspending of RIVA exposed (t:4h) platelets in RIVA naïve plasma (t:0h) restored platelet aggregation following stimulation collagen (n=5, paired *t* test, pre vs post *P*=0.0077, post vs switch *P*=0.0177, pre vs switch *P*=0.5540, Kolmogorov-Smirnov test [KS] showed normal distribution). **E**, Aggregation response following the stimulation with 2 µg collagen in PRP (control n=6 vs RIVA n=6, unpaired *t* test, *P*=0.0298, SW showed normal distribution) and washed platelet (control n=7 vs RIVA n=8, Mann-Whitney test, *P*=0.2186, SW showed no normal distribution) from RIVA treated patients or nonanticoagulated controls. **F**, MEA responses towards ADP and RIVA plasma level for individual patients are shown R^2 is indicated. Correlation between aggregation responses and RIVA plasma levels was calculated by linear regression, R^2 and trendline are shown (*P*=0.006) (**G**) LTA in PRP from apixaban treated patients was performed in the time series analysis for the indicated stimuli (ADP: before apixaban n=6 vs after apixaban n=6 Wilcoxon test, *P*=0.0313, SW showed no normal distribution; collagen: before apixaban n=6 vs after apixaban n=6 Wilcoxon test, *P*=0.0313, SW showed no normal distribution). Data show means ± SD.

response to chronic rivaroxaban treatment compared to controls (Figure 3A, Online Movie II). To investigate later stages of thrombus formation (5 minutes), we took advantage of a pulsatile arterial flow system (average shear 10 dyn/cm^2). Again, thrombus formation on collagen was reduced in whole blood of patients with both chronic (Figure 3B) and single-dose rivaroxaban treatment (Figure 3C). Next, we studied the impact of rivaroxaban on thrombus formation on pathophysiological relevant human atherosclerotic plaque material under laminar flow conditions. As shown in Figure 3D and Online Movie III platelet adhesion and thrombus formation were reduced in the presence of rivaroxaban. Importantly, rivaroxaban antiplatelet effects were also observed in the presence of hirudin (200 U/mL), excluding any potential remaining thrombin mediated effect (Figure 3E). Next, we asked whether rivaroxaban does not only affect platelet adhesion and aggregation

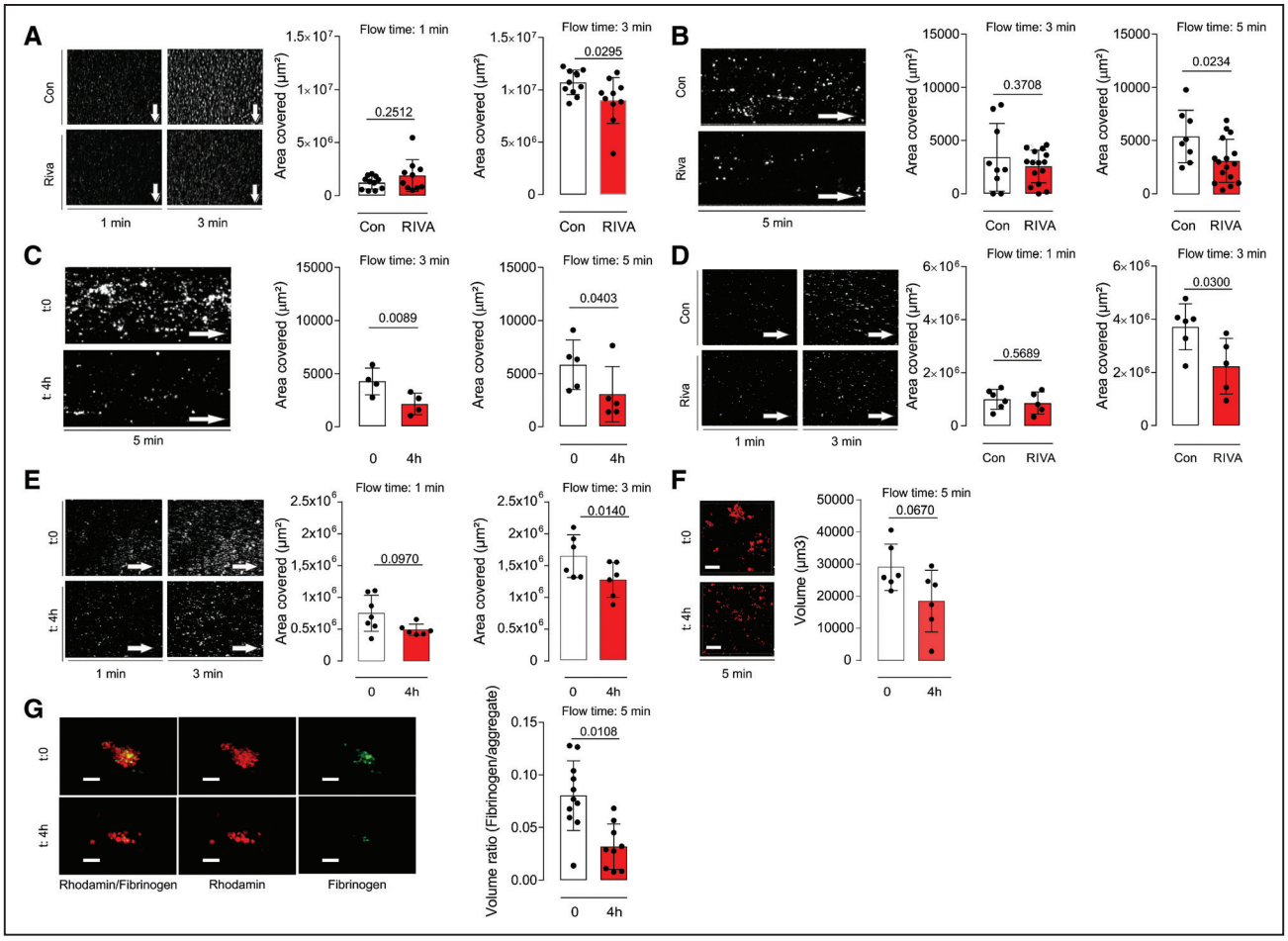

Figure 3. Rivaroxaban (RIVA) reduces platelet adhesion and thrombus formation under flow.
A, Platelet adhesion and thrombus formation in blood from RIVA treated patients or controls were quantified after 1 and 3 min of laminar flow (flow time 1 min—control n=11 vs RIVA n=10, Mann-Whitney test, *P*=0.2512, Shapiro-Wilks [SW] showed no normal distribution; flow time 3 min—control n=11 vs RIVA n=10, Mann-Whitney test, *P*=0.0295, SW showed no normal distribution) or 3 and 5 min of pulsatile flow (**B**) on collagen (flow time 3 min—control n=9 vs RIVA n=15, unpaired *t* test, *P*=0.3708, SW showed normal distribution; flow time 5 min—control n=8 vs RIVA n=15, unpaired *t* test, *P*=0.0234, SW showed normal distribution). **C**, Time series analysis of platelet adhesion and thrombus formation on collagen before and after RIVA treatment (flow time 3 min—before RIVA n=5 vs after RIVA n=5, unpaired *t* test, *P*=0.0089, SW showed normal distribution; flow time 5 min—before RIVA n=5 vs after RIVA n=5, paired *t* test, *P*=0.0403, Kolmogorov-Smirnov test [KS] showed normal distribution) are shown. Representative fluorescent images are shown. **D**, Platelet adhesion and thrombus formation in blood from RIVA treated patients or controls were quantified after 1 and 3 min of laminar flow on human atherosclerotic plaque material coated flow chambers (flow time 1 min—control n=6 vs RIVA n=5, unpaired *t* test, *P*=0.5689, SW showed normal distribution; flow time 3 min—control n=6 vs RIVA n=5, unpaired *t* test, *P*=0.0300, SW showed normal distribution). **E**, Platelet adhesion and thrombus formation in blood from patients pretreated with 200 U/mL hirudin before (t:0h) and after RIVA (t:4h) was quantified after 1 and 3 min of laminar flow on human atherosclerotic plaque material coated flow chambers (flow time 1 min—before RIVA n=6 vs after RIVA n=6, paired *t* test, *P*=0.0970, SW normal distribution; flow time 3 min—before RIVA n=6 vs after RIVA n=6, paired *t* test, *P*=0.0140, SW showed normal distribution). **F**, Quantification of thrombus (red) volume by confocal microscopy before (t:0h) and after RIVA (t:4h) treatment at 5 min of laminar flow in the presence of 200 U/mL hirudin (before RIVA n=6 vs after RIVA n=6, paired *t* test, *P*=0.0670, SW showed normal distribution). **G**, Analysis of thrombus composition (before RIVA n=11 vs after RIVA n=9, paired *t* test, *P*=0.0180, SW showed normal distribution) by quantification of relative fibrinogen (green) to overall thrombus (red) volume by confocal microscopy before (t:0h) and after RIVA (t:4h) treatment at 5 min of laminar flow in the presence of 200 U/mL hirudin. Arrows indicate the direction of flow and represent 120 µm in A, 240 µm in **D** and **E** as well as 10 mm in **B** and **C**. For confocal microscopy pictures scale bares represent 30 µm in **F** and 4 µm in **G**. Representative pictures are shown, all data show means ± SD and significance levels are indicated. All data show means ± SD.

but also aggregate and thrombus volume in the presence of hirudin. Using confocal microscopy, we found a clear trend towards reduced thrombus volume (Figure 3F, Online Movie IV) in rivaroxaban treated patients. Furthermore, the relative amount of incorporated fluorescently labeled fibrinogen was decreased in the presence of rivaroxaban (Figure 3G, Online Movie V) indicating an altered thrombus composition that might contribute to the reduced thrombus stability in vivo. Of note, we observed the same antithrombotic effects on platelet adhesion and thrombus formation after preexposing control blood to otamixaban, a parenteral direct FXa inhibitor (Online Figure III), which again suggests a group-specific effect of FXa antagonists.

FXa-Induces Platelet Activation

Next, we tested the hypothesis that FXa-mediated signaling directly contributes to platelet aggregation and thrombus formation in the absence of thrombin. Hence, we first determined the concentration of catalytically active FXa in our experimental setup. Indeed, we found increased FXa activity in control compared to rivaroxaban treated patients after 6 minutes of aggregation in PRP (Figure 4A). These data show that platelet aggregation comes along with FXa formation, although it is catalytically inhibited in the presence of rivaroxaban.

Given that FXa and thrombin activate vascular wall cells[17] via protease-activated receptors, we addressed the relevance of this signaling pathway in platelets. First, we analyzed early Ca^{2+} signaling by flow cytometry following incubation with a calcium sensitive dye, flou-4. As shown in Figure 4B, FXa triggered early Ca^{2+} signaling similar to TRAP-6, a PAR-1 specific agonist, which both could be inhibited in the presence of the clinically used PAR-1 inhibitor vorapaxar. In addition, we found an augmented surface expression of platelet activation markers P-selectin, CD40L and ICAM (intercellular adhesion molecule)-2, integrin GPIIbIIIA activation as determined by PAC (procaspase activating compound)-1 expression and thromboxane formation in response to FXa (Figure 4C, Online Figure IVA). Furthermore, we found that platelet cargo protein release from α-granules following FXa stimulation occurs to a similar extend as seen on PAR-1 activation by thrombin (Online Figure IVB). To address whether rivaroxaban has some direct effects on platelets, we analyzed platelet intracellular signaling in WP in the absence of coagulation factors and FXa respectively. In the presence of rivaroxaban, we did not find any altered platelet calcium signaling following the stimulation we different agonists besides FXa on a single-cell level (Figure 4D). Next, we investigated the activation of src family kinases following ADP, TRAP, and collagen activation in the absence or presence of 100 pmol rivaroxaban. In line with our calcium data, we did not find profound alteration of src autophosphorylation in WP following stimulation with collagen, TRAP, and ADP stimulation in the presence of rivaroxaban (Online Figure IVC).

To clarify, whether FXa-induced signaling could induce an aggregation response, we performed MEA. As shown in Figure 5A, we found that FXa triggered platelet aggregation in hirudin-anticoagulated blood independently of catalytically active thrombin. Next, we investigated FXa induced aggregation in WP, thereby excluding potential interference with the coagulation system. As depicted in Figure 5B, we found a FXa dose-dependent aggregation response, which was sensitive to rivaroxaban (Figure 5C). These effects were specific to FXa, as other upstream coagulation factors, such as FVa (4 µg/mL), FVIIa (200 µg/mL), and FVIIIa (0.2 µg/mL), did not trigger platelet aggregation responses (Figure 5D). Next, we aimed to identify the underlying molecular mechanism of FXa triggered PAR-1 mediated platelet activation. We determined the FXa cleavage sites within synthetized PAR-peptides that span the extracellular activation domain of platelet expressed PAR-1. Using mass spectrometry, we identified Arg41-Ser42 in PAR-1 as FXa cleavage site (Figure 5E), which is similar to the known thrombin cleavage site. In summary, these data show that FXa directly cleaves PAR-1 leading to physiologically relevant platelet response, such as platelet aggregation.[18] Of note, we did not find any effect of PAR-4 receptor inhibition on platelet aggregation, indicating that PAR-4 does not contribute to platelet aggregation responses in our experimental setup (Online Figure V).

Next, we studied intracellular FXa-induced signaling downstream of PAR-1. Hence, we performed platelet aggregation in the presence of PI3K (phosphoinositide 3-kinase) inhibitor, ROCK (rho-associated, coiled-coil containing protein kinase) inhibitor, and PLC (phospholipase C)-inhibitor. We found that FXa- and TRAP-induced aggregation responses partially depend on PI3K and PLC (Figure 5F). However, the contribution of PLC in both pathways might be overestimated in the setting of platelet aggregation, given that a concomitantly occurring release of platelet granula content induces a second wave of platelet activation through additional receptor cascades.

At sites of plaque rupture in vivo, a complex mixture of soluble and matrix exposed platelet agonists triggers a full-fledged platelet activation in a synergistic manner. Hence, even low doses of FXa might induce a preconditioning of platelets by lowering platelet activation threshold. To follow-up on this concept, we prestimulated platelets with low concentrations of FXa that did not induce aggregation (Online Figure VI) and determined PI3K activity, diacylglycerol, and inositol triphosphate level. As shown in Figure 5G, PI3K activity, diacylglycerol, and inositol triphosphate levels were augmented on FXa in nonaggregatory doses. To address the functional relevance of low-dose FXa preconditioning, we performed aggregation experiments and found that FXa pretreatment increased agonist-induced platelet aggregation (Figure 5H). In line with these findings, we observed an additive effect of FXa preconditioning on Ca^{2+} signaling following the stimulation with collagen and ADP (Figure 5I).

To further elaborate on the finding, that FXa directly stimulated platelet activation via PAR-1, we next conducted experiments using the specific PAR-1 inhibitor vorapaxar. Figure 6A shows that FXa-induced platelet aggregation was indeed blunted by vorapaxar. In addition, we observed that platelet adhesion and thrombus formation under pulsatile flow conditions (Figure 6B) were reduced in the presence of vorapaxar. In analogy to rivaroxaban, vorapaxar treatment reduced aggregation responses towards TRAP-6, ADP, and collagen in PRP (Figure 6C) with only little additive inhibitory effect on FXa stimulation (Figure 6D).

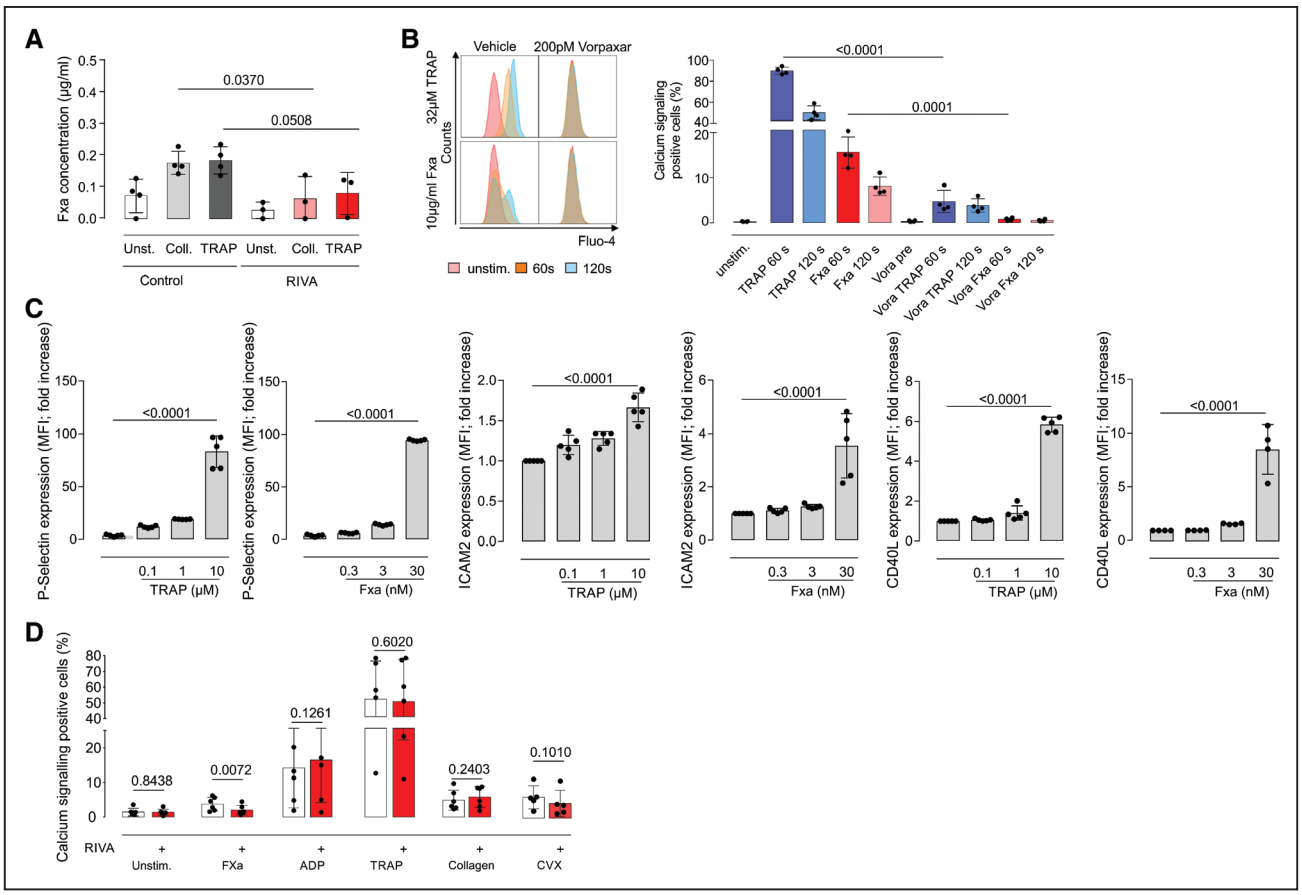

Figure 4. FXa (Factor Xa) induces platelet activation.

A, Concentration of catalytically active FXa during light transmission aggregometry was measured under resting conditions or after stimulation with collagen and TRAP (thrombin receptor-activating peptide) in platelet-rich plasma isolated from rivaroxaban (RIVA) treated patients and controls (unstimulated—control n=4 vs RIVA n=3, unpaired *t* test, *P*=0.24, Shapiro-Wilks [SW] showed normal distribution; collagen—control n=4 vs RIVA n=3, unpaired *t* test, *P*=0.0370, SW showed normal distribution; TRAP—control n=4 vs RIVA n=3, unpaired *t* test, *P*=0.0508, SW showed normal distribution). **B**, Platelet Ca^{2+} signaling was analyzed after stimulation with 10 µg/mL FXa and 12 µmol/L TRAP the absence or presence of PAR (protease-activated receptor)-1 inhibitor vorapaxar (VORA) by flow cytometry (unstimulated—control n=4 vs VORA n=4, unpaired *t* test, *P*=0.44, SW showed normal distribution; TRAP 60 s—control n=4 vs VORA n=4, unpaired *t* test, *P*<0.0001, SW showed normal distribution; SW showed normal distribution; FXa 60 s—control n=4 vs VORA n=4, unpaired *t* test, *P*=0.0001, SW showed normal distribution). **C**, Surface expression of platelet activation marker P-selectin, CD40L and ICAM (intercellular adhesion molecule)-2 was quantified after stimulation with TRAP and FXa using flow cytometry; $P\text{-selectin}_{TRAP}$ n=5—control vs 0.1 µmol/L TRAP vs 1 µmol/L TRAP vs 10 µmol/L TRAP, *P*<0.0001, RM-ANOVA, within testing was performed, Kolmogorov-Smirnov test (KS) showed normal distribution; $P\text{-selectin}_{FXa}$ n=5—control vs 0.3 nmol/L FXa vs 3 nmol/L FXa vs 30 nmol/L FXa, *P*<0.0001, RM-ANOVA, within testing was performed, KS showed normal distribution; $ICAM\ 2_{TRAP}$ (fold of unstimulated control) n=5—control vs 0.1 µmol/L TRAP vs 1 µmol/L TRAP vs 10 µmol/L TRAP, *P*<0.0001, RM-ANOVA, within testing was performed, KS showed normal distribution; $ICAM\ 2_{FXa}$ (fold of unstimulated control) n=5—control vs 0.3 nmol/L FXa vs 3 nmol/L FXa vs 30 nmol/L FXa, *P*<0.0001, RM-ANOVA, within testing was performed, KS showed normal distribution; $CD40L_{TRAP}$ (fold of unstimulated control) n=5—control vs 0.1 µmol/L TRAP vs 1 µmol/L TRAP vs 10 µmol/L TRAP, *P*<0.0001, RM-ANOVA, within testing was performed, KS showed normal distribution; $CD40L_{FXa}$ (fold of unstimulated control) n=4—control vs 0.3 nmol/L FXa vs 3 nmol/L FXa vs 30 nmol/L FXa, *P*<0.0001, Friedman test, within testing was performed, n too small to pass normality testing. All data show means±SD. **D**, Platelet Ca^{2+} signaling was analyzed after stimulation with 10 µg/mL FXa, 20 µmol/L ADP, 12 µmol/L TRAP, 50 µg/mL collagen, and 50 ng/mL CVX in the absence or presence of 100 pmol/L RIVA by flow cytometry (unstimulated—control n=6 vs RIVA n=6, Wilcoxon test, *P*=0.8438, SW showed no normal distribution; FXa—control n=6 vs RIVA n=6, paired *t* test, *P*=0.0072, SW showed normal distribution; ADP—control n=6 vs RIVA n=6, paired *t* test, *P*=0.1261, SW showed normal distribution; TRAP—control n=6 vs RIVA n=6, paired *t* test, *P*=0.6020, SW showed normal distribution; collagen—control n=6 vs rivaroxaban n=6, paired *t* test, *P*=0.2403, SW showed normal distribution; convulxin—control n=5 vs RIVA n=5, ratio paired *t* test, *P*=0.1010, SW showed normal distribution). Data show means ± SD.

Platelet Activation Under Low-Dose Rivaroxaban Treatment

Against the background of current clinical studies that reported beneficial effects of low-dose rivaroxaban on top of antiplatelet therapy following acute coronary syndrome or in patients with stable coronary artery disease, we next investigated whether a low-dose rivaroxaban regime had similar antiplatelet effects. Hence, we analyzed control blood before and 4 hours after rivaroxaban (2.5 mg) intake. As shown in Figure 7A, we did not find any differences in aggregation responses in

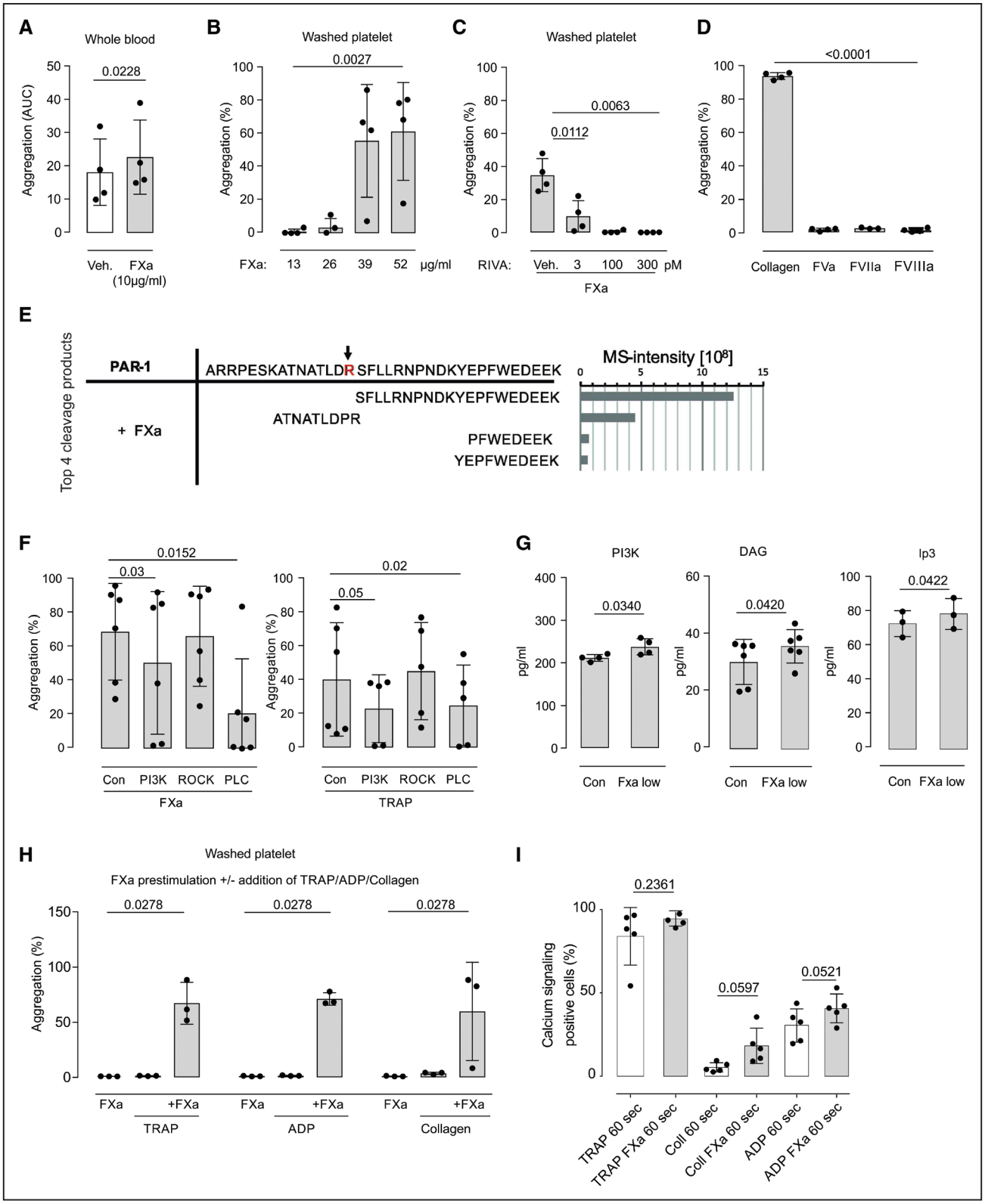

Figure 5. FXa (factor Xa) triggers platelet aggregation.

A, Whole blood aggregation was quantified after stimulation with 10 µg/mL FXa (vehicle n=4 vs FXa n=4, paired *t* test, *P*=0.0228, Shapiro-Wilks [SW] showed normal distribution). **B**, Platelet aggregation responses of washed platelets were analyzed using light-transmittance aggregometry (LTA) following stimulation with the indicated concentrations of FXa (FXa stimulation n=4–13 µg/mL FXa vs 26 µg/mL FXa vs 39 µg/mL FXa vs 52 µg/mL FXa, Friedman test, *P*=0.0027, within testing was performed, n too small to pass normality testing) and (**C**) in the absence or presence of rivaroxaban (RIVA; FXa stimulation n=4–control vs 3 pmol/L RIVA vs 100 pmol/L RIVA vs 300 pmol/L RIVA; Friedmann test, *P*=0.0063, within testing was performed; control vs 3 pmol/L RIVA, paired *t* test, *P*=0.0112, Kolmogorov-Smirnov test [KS] showed normal distribution). (*Continued*)

whole blood towards ADP, TRAP, and human atherosclerotic plaque or in PRP following stimulation with ADP or collagen (Figure 7B). Next, we analyzed thrombus formation under laminar arterial flow conditions on collagen, but we did not observe any impact of rivaroxaban on surface coverage (Figure 7C). Using flow cytometry, we quantified single platelet activation by determining the percentage of P-selectin positive platelets. Although platelet activation under basal condition was unchanged, we observed an attenuated platelet reactivity following FXa, TRAP, and collagen stimulation (Figure 7D). In summary, these data suggest that rivaroxaban's antiplatelet effects are dose-dependent and are attenuated but still detectable following a single low-dose treatment.

DISCUSSION

In this study, we addressed the mechanisms underlying the reduced frequency in ischemic events observed in patients receiving rivaroxaban. We found that platelet activation and aggregation, as well as thrombus formation under arterial flow conditions were attenuated under rivaroxaban treatment in vitro and in vivo. Our data highlight a so far underestimated thrombin independent FXa-induced platelet activation through PAR-1 receptor.

Although the central role of FXa as key propagator of coagulation that multiplies thrombin formation during primary hemostasis is extensively investigated, its impact on platelet activation is less well understood. During the early phase of arterial thrombus formation FXa generation takes place at the surface of procoagulant platelets and the injured activated endothelium.[10,11] Exposed tissue factor then drives FVIIa and subsequently FXa activation around the lesion site.[19,20]

To investigate the pathophysiological significance of FXa-mediated effects on platelets and subsequently the role of rivaroxaban during these early steps of atherothrombosis, we established an experimental in vitro setup, that allowed us to test the effects of FXa generation that are independent of thrombin and its catalytic activity. Using this approach, we identified direct platelet activation after addition of purified FXa as well as in response to de novo generated FXa. Subsequently, we revealed PI3K and PLC as involved downstream pathways,[21] both known to contribute to platelet activation.[22,23] To do justice to the more complex situation at sites of plaque rupture in vivo where platelets encounter a mixture of different agonists with varying localized agonist concentration, we investigated platelet responses to low-dose FXa. Indeed we found that low-dose FXa treatment enhanced intracellular PI3K activity as well as inositol triphosphate and diacylglycerol level and increased calcium signaling. This indicates a platelet preconditioning, which contributes to platelet aggregation and activation which was already described in the setting of shear forces signaling.[24]

In this study, we dissected rivaroxaban's so far unknown antiplatelet from its anticoagulatory effect. Catalytically active thrombin is one of the most potent platelet activators triggering full-fledged platelet activation and thrombus formation through activation of PAR-1 and PAR-4. However, in our setting, FXa-mediated PAR-4 signaling did not significantly contribute to platelet aggregation responses, which is in line with earlier data.[25,26] In contrast, vorapaxar, a PAR-1 specific inhibitor, as well as rivaroxaban, could inhibit Ca^{2+}-signaling on a single platelet level, platelet aggregation, and thrombus formation under flow conditions. The pathophysiological significance of thrombin independent PAR-1 activation through receptor cleavage[27] during arterial thrombosis in vivo and platelet aggregation in vitro was shown for MMP (metalloproteinase)-1. In our study, we identified

Figure 5 Continued. **D**, Aggregation responses of washed platelets was determined after stimulation with 2 µg/mL collagen or coagulation factors FVa (4 µg/mL), FVIIa (200 µg/mL), and FVIIIa (0.2 µg/mL), respectively (platelet aggregation response—collagen vs FVa vs FVIIa vs FVIIIa, Friedmann test, $P<0.0001$, within testing was performed, KS showed normal distribution). **E**, Mass spectrometry analysis of PAR (protease-activated receptor)-1 peptide cleavage products following the incubation with FXa. Representative results of 2 independent experiments are shown. **F**, Platelet signaling following aggregation responses (LTA) triggered by FXa or TRAP (thrombin receptor-activating peptide) was analyzed after inhibition of PI3K (phosphoinositide 3-kinase), PLC (phospholipase C), or ROCK (rho-associated, coiled-coil containing protein kinase) activity by specific inhibitors. Platelet aggregation responses are shown (LTA with FXa n=6—control vs PI3K inhibitor vs ROCK inhibitor vs PLC inhibitor, RM-ANOVA, $P=0.0399$, within testing was performed; control vs PI3K inhibitor, paired *t* test, $P=0.0342$; KS showed normal distribution; LTA with TRAP n=5—control vs PI3K inhibitor vs ROCK inhibitor vs PLC inhibitor, RM-ANOVA, $P=0.0211$, within testing was performed; control vs PI3K inhibitor, paired *t* test, $P=0.0270$; KS showed normal distribution). **G**, Platelet preconditioning by low-dose FXa stimulation that does not induce a significant platelet aggregation was quantified by determination of PI3K activity as well as diacylglycerol (DAG) and inositol triphosphate (IP3) level using ELISA assay (IP3 n=3—control vs FXa low, Wilcoxon test, $P=0.0340$, n too small to pass normality testing; PI3K n=4—control vs FXa low, Wilcoxon test, $P=0.0420$, n too small to pass normality testing; DAG n=4—control vs FXa low, IP3 vs FXa low, Wilcoxon test, $P=0.0422$, n too small to pass normality testing). **H**, Aggregation responses of washed platelets were determined after prestimulation with FXa (10 µg/mL) followed by low-dose stimulation with the indicated agonists. Concentrations of the respective agonist were determined in individual dose-response curves (not shown) to achieve a submaximal aggregation response (TRAP n=3—FXa control vs TRAP vs TRAP + FXa, Friedmann test, $P=0.0278$, n too small to pass normality testing; ADP n=3—FXa control vs ADP vs ADP + FXa, Friedmann test, $P=0.$ 0278 n too small to pass normality testing; collagen n=3—FXa control vs collagen vs collagen + FXa, Friedmann test, $P=0.0278$, n too small to pass normality testing). **I**, Flow cytometric single-cell analysis of calcium signaling of washed platelets was determined after prestimulation with FXa (10 µg/mL) followed by stimulation with the indicated agonists (12 µmol/L TRAP—control n=5 vs FXa n=5, ratio paired *t* test, $P=0.2361$, SW showed normal distribution; 50 µg/mL collagen—control n=5 vs FXa n=5, ratio paired *t* test, $P=0.0597$, SW showed normal distribution; 20 µmol/L ADP—control n=5 vs FXa n=5, ratio paired *t* test, $P=0.0521$, SW showed normal distribution; all data show means ± SD).

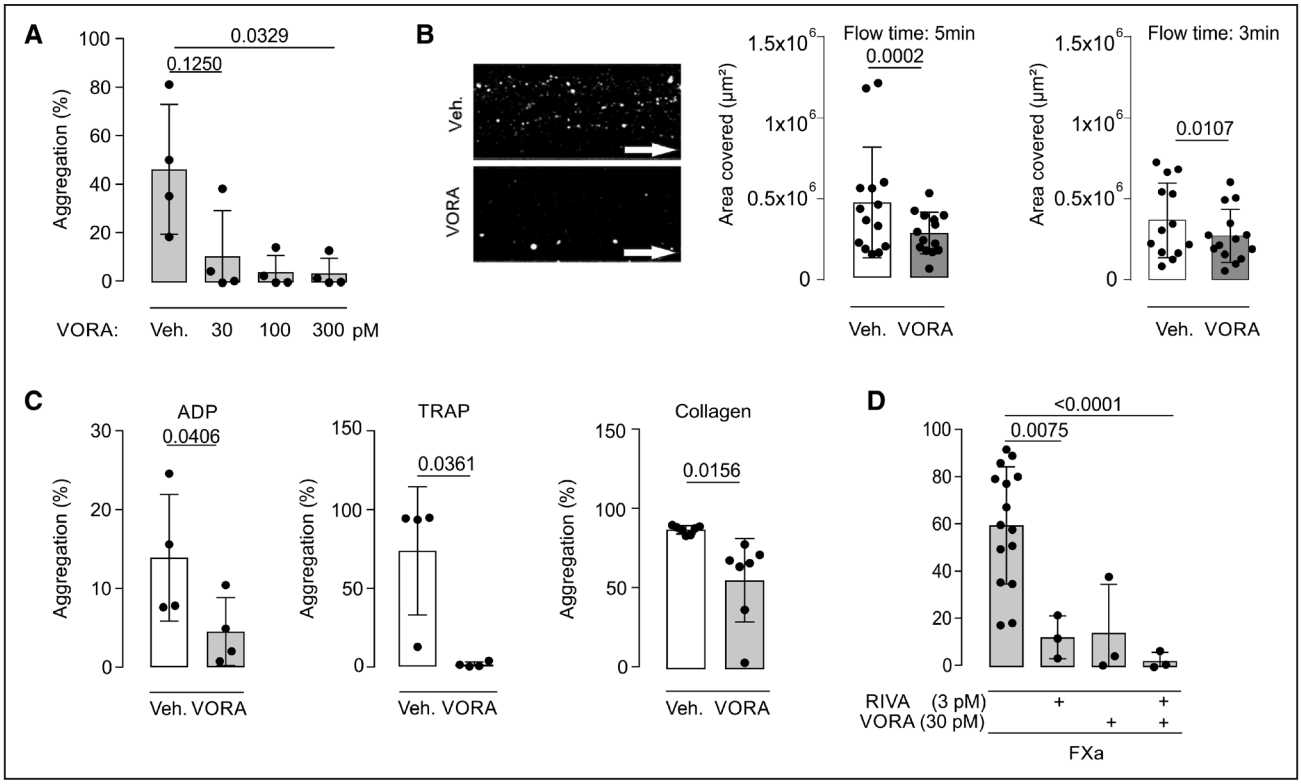

Figure 6. FXa (factor Xa) triggers platelet activation through PAR (protease-activated receptor)-1.
A, FXa-induced platelet aggregation was analyzed in the presence or absence of PAR-1 inhibitor vorapaxar (VORA) as measured by light transmission aggregometry (LTA with VORA and FXa n=4—vehicle vs 30 pmol/L VORA vs 100 pmol/L VORA vs 300 pmol/L VORA, all: Friedmann test, *P*=0.0329; vehicle vs 30 pmol/L VORA: Wilcoxon test, *P*=0.1250, n too small to pass normality tests). **B**, Platelet adhesion and thrombus formation in collagen coated flow chambers in the presence or absence of VORA was analyzed (flow time 5 min—vehicle n=14 vs VORA n=14, Wilcoxon test, *P*=0.0002, Shapiro-Wilks [SW] did not show normal distribution; flow time 3 min—vehicle n=13 vs VORA n=14, paired *t* test, *P*=0.0107, SW showed normal distribution). **C**, Platelet aggregation after stimulation with TRAP (thrombin receptor-activating peptide), ADP, and collagen was analyzed in presence of VORA (ADP: vehicle [n=4] vs VORA [n=4]) paired *t* test, *P*=0.0406, SW showed normal distribution; TRAP: vehicle (n=4) vs VORA (n=4), Wilcoxon test, *P*=0.0361, SW showed no normal distribution; collagen: vehicle (n=7) vs VORA (n=7), Wilcoxon test, *P*=0.0156, SW showed no normal distribution. **D**, Platelet aggregation by LTA was quantified after addition of VORA, RIVA or both antagonists following stimulation with FXa (LTA with FXa, VORA and RIVA—control n=15 [SW showed normal distribution] vs 3 pmol/L RIVA n=3 [n too small to pass normal distribution] vs 30 pmol/L VORA n=3 [n too small for normally distribution] vs 3 pmol/L RIVA + 30 pmol/L VORA n=3 [n too small for normally distribution], control vs VORA+RIVA, Kolmogorov-test, *P*<0.0001, *f* test revealed that variances were not equal; control vs RIVA, *U* test, *P*=0.0075). Data shows means ± SD.

Arg41-Ser42 in PAR-1 as FXa cleavage sites, which is also known as thrombin cleavage sites.[28]

The antithrombotic potential of inhibiting PAR-1 signaling was shown in different clinical studies. PAR-1 inhibition by vorapaxar on top of single or dual antiplatelet therapy proofed to efficiently reduce thrombotic events in patients with stable coronary artery disease or acute coronary events.[29–31] However, this improved antithrombotic efficiency came along with an increased risk for clinically relevant hemorrhage.[29] We found that both drugs, either vorapaxar or rivaroxaban efficiently inhibited FXa-mediated platelet activation. Interestingly, in comparison to vorapaxar a similar benefit-to-harm ratio was found in recent clinical studies that combined low-dose rivaroxaban (2.5 mg bid) on top of antiplatelet therapy, which reported reduced MI rates[8] or lowered cardiovascular mortality[9] while increasing bleeding risks.

Our data show that rivaroxaban treatment of WP does not affect platelet activation in the absence of FXa. We show that upon platelet activation, plasma-derived coagulation factors are crucial to drive FXa de novo synthesis and subsequently platelet activation. Hence, our study suggests that rivaroxaban's antiplatelet effects do not require a direct binding of rivaroxaban to platelets but rather inhibit plasma dependent FXa activity. This contrasts a recent study suggesting a direct interaction between platelet GPVI and rivaroxaban that attenuates convulxin induced platelet responses.[32] However, in our study, we did not find a direct effect of rivaroxaban single-cell Ca^{2+}, activation on stimulation with convulxin or collagen. Of note, the latter is the physiological relevant agonist in the context of arterial thrombosis. Furthermore, we found that rivaroxaban naïve plasma could restore reduced platelet aggregation responses upon rivaroxaban treatment, underlining the plasma dependency of rivaroxaban's antiplatelet effect. Hence, further studies analyzing the structural features of potential direct rivaroxaban platelet interactions are required.

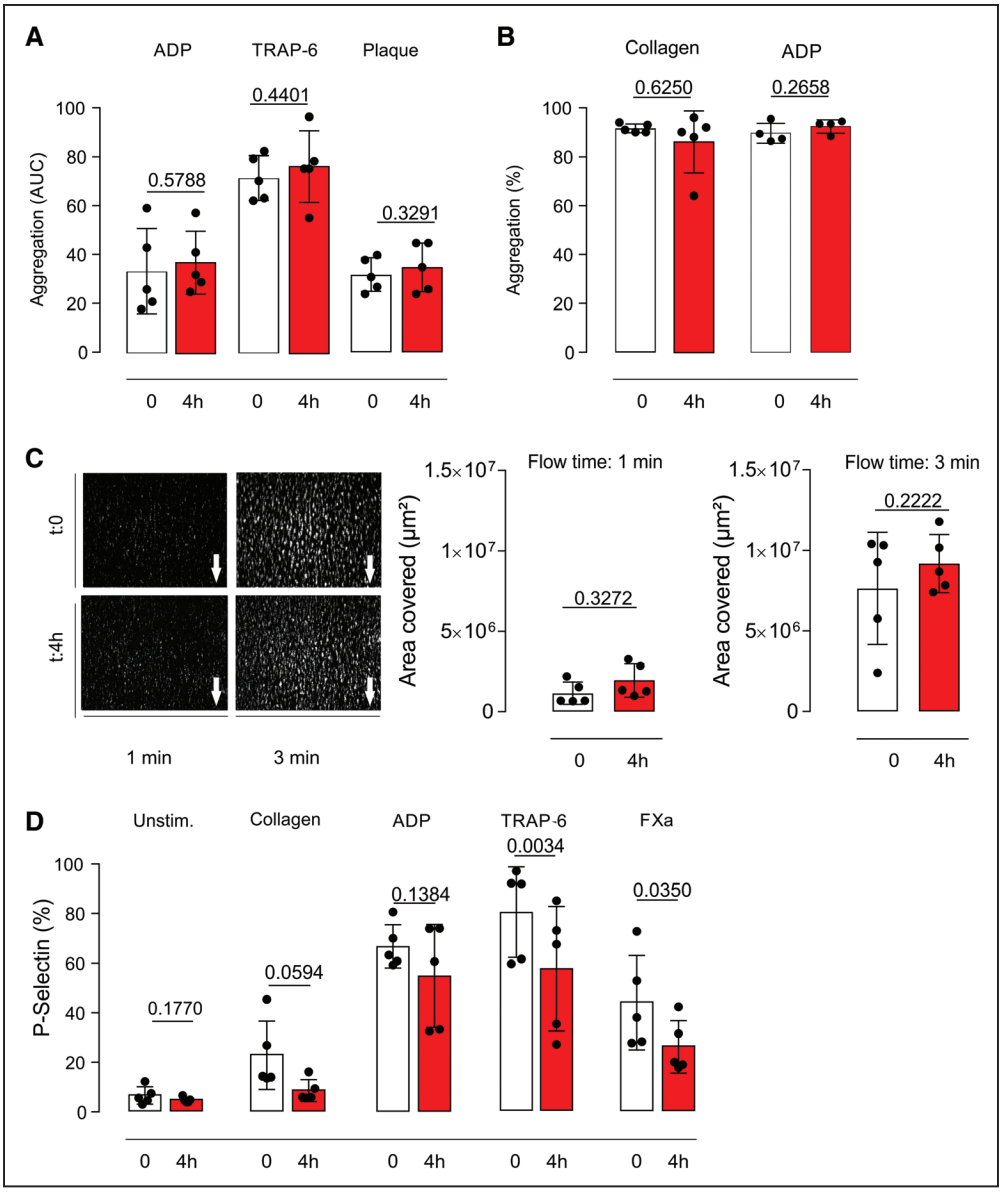

Figure 7. Platelet activation under low-dose rivaroxaban (RIVA) treatment.
A, Whole blood multiple-electrode aggregometry before and 4 h after single low-dose RIVA treatment (2.5 mg) upon stimulation with 5 µmol/L ADP, 12 µmol/L TRAP (thrombin receptor-activating peptide) and human atherosclerotic plaque was analyzed (n=5 in each group; ADP: before vs after, *P*=0.5788; TRAP: before vs after, *P*=0.4401; plaque: before vs after, *P*=0.3291; paired *t* tests, Kolmogorov-Smirnov test [KS] test showed normal distribution). **B**, Light transmission aggregometry (LTA) before and 4 h after single low-dose RIVA treatment (2.5 mg) in platelet-rich plasma (PRP) upon stimulation with 2 µg/mL collagen (before RIVA n=5 vs after RIVA n=5, Wilcoxon test, *P*=0.6250, Shapiro-Wilks [SW] did not show normal distribution) and 5 µmol/L ADP (before RIVA n=4 vs after RIVA n=4, paired *t* test, *P*=0.2658, SW showed normal distribution) was quantified. **C**, Time series analysis of platelet adhesion and thrombus formation on collagen before and after low-dose RIVA treatment (flow time 1 min—before RIVA n=5 vs after RIVA n=5, paired *t* test, *P*=0.3271, SW showed normal distribution; flow time 3 min—before RIVA n=5 vs after RIVA n=5, paired *t* test, *P*=0.2222, SW showed normal distribution). Platelet adhesion and thrombus formation on collagen was quantified after 1 and 3 min of laminar flow. Arrows indicate the direction of flow and represent 120 µm (all data show means ± SD). Representative fluorescent images are shown. **D**, Platelet activation by quantification of P-selectin positive platelets was determined by flow cytometry following the stimulation with the indicated agonists before and after RIVA intake (unstimulated [n=4], *P*=0.1770; 4 µg/mL collagen [n=4], *P*=0.0594; 20 µmol/L ADP [n=5], *P*=0.1384; 10 µmol/L TRAP [n=5], *P*=0.0034; 0.45 µg/mL FXa [n=5], *P*=0.0350; paired *t* tests, KS test showed normal distribution). Data show means ± SD.

We show that already a single dose of either 20 or 2.5 mg of orally ingested rivaroxaban attenuated platelet activation though in a dose-dependent manner. For the low-dose regime (ie, 2.5 mg) a recent study reported some antiplatelet effects for patients under additional dual antiplatelet therapy,[8,33] indicating that rivaroxaban's antiplatelet effects are sustained even in the presence of dual antiplatelet therapy. This is of interest as even minor changes in platelet reactivity (ie, reduced P-selectin expression level) are associated with a reduced

composite end point of cardiovascular death, MI, and stent thrombosis in a cohort with recent MI under dual antiplatelet therapy.[34] Against the background of our dose-response experiments, we choose a rivaroxaban concentration of 100 pmol for in vitro experiments. This concentration is significantly below achieved rivaroxaban peak level that range between 184 and 343 ng/mL (422–787 pmol/L) in patients[35,36] underscoring the translational implications and significance of our findings.

Several earlier studies[37–40] investigated platelet function in the context of different FXa inhibiting nonvitamin K anticoagulants but reported contradicting results. Recently, Nehaj et al[38] describe a reduced TRAP-induced platelet aggregation under rivaroxaban treatment, although the underlying mechanism remains elusive. On the contrary, enhanced platelet reactivity in rivaroxaban treated patients has been recently described as well.[40] Additionally, it has been unknown whether the observed effects are substance or rather group specific. Our data revealed similar antiplatelet effects for apixaban and otamixaban and hence support the concept of an FXa inhibitor specific group effect. Indeed, within the ARISTOTLE trial (Apixaban for Reduction in Stroke and Other Thromboembolic Events in Atrial Fibrillation) comparing vitamin K antagonist versus apixaban for stroke prevention, a nonsignificant risk reduction of MI was observed within the apixaban arm.[4] Furthermore, a reduced risk for MI came up in a large network meta-analysis and register data that reported reduced risk of MI under rivaroxaban and apixaban compared with warfarin.[41,42] Unlike FXa, FIIa inhibition associated with an increased platelet reactivity due to an altered GPIbα thrombin interaction in the presence of shear forces[13] and enhanced platelet PAR-1 surface expression as the receptor degradation was reduced.[43,44] Whether this mechanism contributes to the mild numerical increase in the frequency of MI under oral thrombin inhibition is still under debate.[42,45,46]

Our in vivo animal experiments identified an antithrombotic effect with a reduced thrombus stability under rivaroxaban, which is in line with an earlier study that reported a reduced burden of thrombosis in a different model of arterial thrombosis.[47] Importantly, we observed a similar phenotype with a reduced thrombus stability on treatment with aspirin in the same model[12] in an earlier study. Although the interpretation of these findings is limited giving that dissecting rivaroxaban's antiplatelet effects from its anticoagulatory function in vivo is difficult as both mechanisms will occur (almost) simultaneously at sites of vessel injury. To overcome this short coming, we applied 2 different approaches. First, we analyzed platelet activation towards pathophysiological highly relevant human atherosclerotic plaque material under rivaroxaban treatment and observed the same antiplatelet effect on aggregation and thrombus formation under flow conditions. High-resolution confocal microscopy revealed that rivaroxaban treatment reduces thrombus volume and changes thrombus composition by attenuated fibrinogen in cooperation. Again, these effects were independent of thrombin as experiments were performed in hirudin spiked blood. Second, histological analysis of the carotid thrombi from in vivo experiments revealed reduced platelet coverage within the thrombus which is similar to coronary thrombus aspirates in patients under aspirin therapy,[48] hence supporting the antiplatelet effects of rivaroxaban in vivo.

This study has some limitations. First, the sample size was limited and we recruited also nonanticoagulated control patients without AF. Moreover, gender rate and comorbidities were not balanced between groups in particular within the cross-sectional cohort. To overcome potential intergroup differences on platelet reactivity due to variations in baseline characteristics or due to the underlying arrythmia,[15,49] we complemented our data with data from time series experiments from the same individuals and observed the same antiplatelet effect. Due to organizational constraints and resource limitations not all experiments could be performed for all individuals. However, a particular strength of this study is that all key experiments were performed independently of each other at the 2 study sites. In our experiments, we could show that PLC reduced the effect of FXa on platelet aggregation. However, PLC inhibition was investigated in a platelet aggregation setup, where platelet activation is boosted subsequently to platelet granula release and autocrine signaling events. Hence, PLC inhibition within an aggregation setting will not only interfere with FXa induced but also secondary, autocrine-induced signaling events. In contrast, Ca^{2+} signaling was determined on single-cell level, reflecting a more specific FXa effect.

In conclusion, our study revealed an antiplatelet mechanism of rivaroxaban. Rivaroxaban inhibits direct platelet activation by FXa via PAR-1. Together with its well-known potent anticoagulatory capacity, this antiplatelet effect may contribute to reduced ischemic events and improved outcome in rivaroxaban treated patients.[50–54]

ARTICLE INFORMATION

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Disclosures

None.

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