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Identification of coagulation factor XI as a ligand for platelet apolipoprotein E receptor 2 (ApoER2)

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SUMMARY

Objective—Factor XI (FXI) promotes hemostasis and thrombosis through enhancement of thrombin generation, and has been shown to play a critical role in the formation of occlusive thrombi in arterial injury models. The aim of this study was to investigate the mechanisms governing interactions between FXI and platelets.

Methods and Results—Platelet adhesion to immobilized FXI was abrogated in the presence of the low-density lipoprotein (LDL) receptor antagonist, receptor-associated protein (RAP); soluble recombinant Apolipoprotein E receptor 2 (ApoER2); or the LDL-binding domain 1 or 2 of ApoER2. FXI supported wild-type murine platelet binding; in contrast, ApoER2-deficient murine platelets did not adhere to FXI. In the presence of shear, platelet aggregates formed on FXI or activated FXI (FXIa) surfaces, while the presence of RAP, binding domain 1 of ApoER2 or an anti-GPIbα mAb blocked platelet adhesion to FXI or FXIa under shear. Soluble FXI bound to immobilized ApoER2' with an affinity of 61 nM.

Conclusions—This study has identified apolipoprotein E receptor 2 (ApoER2, LRP8), a member of the LDL receptor family, as a platelet receptor for FXI. The interaction of FXI with other cell types that express ApoER2 remains to be explored.

Keywords

platelets; GPIb; apolipoprotein E receptor 2; LRP8; factor XI

INTRODUCTION

Factor XI (FXI) is a 160 kDa glycoprotein that participates in the intrinsic blood coagulation pathway and contributes to hemostasis. Activated FXI (FXIa) is a serine protease generated by cleavage of FXI at the Arg^{369}-Ile^{370} bond by activated factor XII, thrombin or via

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DISCLOSURES
None

autocatalytic activation.[1] FXIa contributes to sustained thrombin generation via activation of factor IX (FIX).[2] Inherited FXI deficiency causes a mild bleeding diathesis and is protective against ischemic stroke,[3, 4] while an elevated FXI plasma level is an independent risk factor for thrombotic diseases such as deep vein thrombosis.[5] Consistent with these observations, FXI plays a critical role in experimental thrombus growth in rabbits, mice and primates.[6–10]

FXI circulates as a disulfide-linked homodimer in complex with plasma high molecular weight kininogen (HK). FXI shares high sequence homology (58% amino acid identity) with the functionally-distinct plasma protein prekallikrein, which also circulates in complex with HK.[11, 12] While the serine protease domain of each FXI subunit is similar to catalytic domains for other coagulation proteases, the non-catalytic portion contains four apple domains (A1 to A4), a feature shared only with prekallikrein.[12, 13] The FXI A3 domain has been shown to contain binding sites for FIX and for the platelet receptor glycoprotein Ib-IX-V (GPIb).[14, 15] FXI binding has been localized to the leucine-rich repeat (LRR) sequences on the NH_2-terminal globular domain of GPIbα, at a site distinct from the anionic thrombin-binding domain of GPIbα.[16, 17] It is unknown if FXI-platelet binding is solely mediated by GPIbα or if other platelet receptor(s) exist that can support interactions with FXI.

GPIbα has been shown to form a complex on the platelet surface with apolipoprotein E receptor 2 (ApoER2, LRP8),[18–20] a member of the low density lipoprotein (LDL) family of receptors. ApoER2 initiates intracellular signaling through the adaptor protein disabled-1 (Dab-1) in platelets.[21] The extracellular domain of ApoER2 consists of three regions: (i) the type A-binding repeats of LDL-binding domains displaying a negatively-charged surface that are responsible for receptor-ligand interactions; (ii) type B repeats, which are homologous to regions in the epidermal growth factor precursor; and (iii) the protein stack of O-linked sugar domains that separate the LDL-binding domains from the cellular surface. We have recently shown that platelet and leukocyte ApoER2 mediate interactions with the anticoagulant serine protease, activated protein C.[22, 23] Here, we present the first evidence that identifies FXI as a ligand for ApoER2.

MATERIALS & METHODS

Reagents

Plasma-derived FXI, FXIa and FXa were purchased from Haematologic Technologies, Inc. (Essex Junction, VT, USA), fibrinogen and high molecular weight kininogen were from Enzyme Research Laboratories, Inc. (South Bend, IN, USA), fibrillar collagen was from Chrono-Log Corporation (Havertown, PA, USA), and AK2 was from GeneTex, Inc. (San Antonio, TX, USA). Soluble GPIbα, ApoER2' (a splice variant of ApoER2 lacking LDL-binding domains 4, 5 and 6), low density lipoprotein (LDL)-binding domains 1 and 2 of ApoER2, plasma-derived β2GPI and dimeric β2GPI were cloned, expressed and purified as described.[19, 24–27] HRP-labeled anti-FXI antibody was purchased from diaPharma (West Chester, OH). Mouse anti-FXI mAb has been described previously [28] and HRP-conjugated rabbit-anti-mouse IgG was from Dako (Glostrup, Denmark). All other reagents were from Sigma-Aldrich, Inc. (St. Louis, MO, USA) or previously named sources.[29, 30]

Preparation of Human and Murine Platelets and Red Blood Cells

Human venous blood was drawn by venipuncture from healthy volunteers into sodium citrate (final concentration 0.38% vol/vol) and acid/citrate/dextrose (ACD, 10% vol/vol). Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 200 × *g* for 20 min. The platelets were then isolated from PRP by centrifugation at 1000 × *g* for 10 min in the presence of prostacyclin (0.1 μg/ml). Following centrifugation steps, purified human platelets were resuspended in modified Tyrode buffer (129 NaCl mM, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12

mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$; pH 7.3) as previously described.[29]

Following the removal of PRP, red blood cells were pelleted at 2000 × *g* for 10 min at 25 C. Red blood cells were subsequently washed three times with red blood cell buffer (10 mM HEPES, 140 mM NaCl, 5 mM glucose, pH 7.4) as previously described.[29]

Mice deficient in ApoER2 were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). Murine blood was drawn from CO_2 terminally-anesthetized mice into ACD at a ratio of 1:10. PRP was obtained by centrifugation at 200 × *g* for 6 min. Washed platelets were prepared via centrifugation of PRP at 1000 × *g* in the presence of prostacyclin (0.1 μg/ml) for 6 min. The pellet was resuspended in modified Modified Tyrode buffer to the desired platelet count. All animals were maintained using housing and husbandry in accordance with institutional regulations.

Static adhesion assays

Glass coverslips were incubated with a 50 μg/ml solution of FXI, FXIa or fibrinogen for 1 hr at room temperature. Surfaces were then blocked with denatured fatty acid-free bovine serum albumin (BSA, 5 mg/ml) for 1 hr and washed with phosphate-buffered saline (PBS). Purified human platelets (2×10^7/ml) were incubated on protein-coated coverslips at 37°C for 45 min. Platelet spreading was imaged using Köhler illuminated Nomarski differential interference contrast (DIC) optics with a Zeiss 63× oil immersion 1.40 NA plan-apochromat lens on a Zeiss Axiovert 200M microscope (Carl Zeiss, Thornwood, NY). The degree of adhesion was computed using Image J software.[31]

Solid phase binding assays

Soluble ApoER2' (sApoER2'; 5 μg/ml) was coated on Corning Costar 2595 protein assay plates as previously described.[22] Coated wells were blocked with BSA for 1 hr at room temperature. Wells were washed 4 times with washing buffer (PBS with 0.1% tween-20). Prescribed dilutions of human FXI (0–200 nM) in the absence or presence of 40 μg/ml RAP were added to the wells for 1 hr at room temperature. In order to detect ApoER2'-bound FXI, plates were incubated with a mouse anti-human FXI mAb (1 μg/ml) for 1 hr prior to washing and incubation with an HRP-labeled rabbit anti-mouse antibody. The assay was developed by addition of TMB (100 μl/well) followed by acid stop with 1M H_2SO_4, and changes in absorbance at 450 nm were measured. The apparent K_d value was obtained from the binding data using Prism 4 software (GraphPad Software, Inc., San Diego, CA).

Flow adhesion assays

Glass capillary tubes were incubated for 1 hr at room temperature with a solution of 50 μg/ml of either FXI or FXIa. Tubes were then washed with PBS, followed by incubation with 5 mg/ml denatured BSA for 1 hr. Tubes were then incorporated into a flow system on the stage of a Zeiss Axiovert microscope (Carl Zeiss, Thornwood, NY). Reconstituted blood (autologous packed red blood cells reconstituted with washed platelets in modified Tyrode buffer at a final concentration of 3×10^8 platelets/ml, 50% vol/vol) was perfused through the chamber for 3 min at a wall shear rate of 600 s^{-1}. Platelet interactions were imaged in real time with a Zeiss AxioCam.

Analysis of data

Data are shown as means ± SEM. Statistical significance of differences between means was determined by ANOVA. If means were shown to be significantly different, multiple

comparisons were performed by the Tukey test. Probability values of $P < 0.05$ were selected to be statistically significant.

RESULTS

Platelets bind to and spread on factor XI

We sought to investigate the ability of FXI to support platelet adhesion and spreading. The interaction of purified platelets with surface-immobilized FXI was monitored in real-time using DIC microscopy. As shown in Fig. 1A, adhesion of platelets to immobilized FXI was followed by a series of characteristic changes in platelet morphology over a 20 minute time course resulting in the formation of large, sheet-like lamellipodia. Consistent with previous reports, surface-immobilized fibrinogen was also able to support platelet adhesion and spreading (Fig 1A). Fluorescent labeling of the actin cytoskeleton revealed that stress fibers were formed in fully spread platelets on FXI (Fig. 1B). Real-time imaging of platelets loaded with the calcium-reporter dye, Oregon Green BAPTA 1-AM, showed that platelets interacting with FXI demonstrated an initial Ca^{2+} release followed by a delay of up to 5 minutes, after which a further increase in intracellular calcium occurred that oscillated over 3–7 minutes before declining (Fig. 1B). On fibrinogen, platelets displayed an initial Ca^{2+} spike that oscillated in a descending manner over the 12 minutes shown (Fig. 1B). A similar degree of platelet spreading, actin reorganization, and intracellular calcium mobilization was recorded for platelets on immobilized FXIa (Supplemental Fig. 1).

We next aimed to determine the intracellular mechanisms that facilitate platelet spreading on FXI. Presence of an ADP scavenger (apyrase, 2 U/ml) with a cyclooxygenase inhibitor (indomethacin, 10μM) reduced platelet adhesion and spreading on FXI surfaces (Supplemental Fig. 2). Treatment of platelets with inhibitors to Src kinases (PP2, 20 μM), PI3-kinases (wortmannin, 100 nM) or an intracellular Ca^{2+} chelator (BAPTA-AM, 10 μM) inhibited platelet spreading on immobilized FXI (Supplemental Fig. 2). Similar results were seen on FXIa surfaces (Supplemental Fig. 2).

ApoER2 mediates platelet adhesion to FXI

FXI has been shown to bind specifically and reversibly to high affinity sites on the platelet surface. To characterize the molecular mechanisms of platelet-FXI binding, adhesion assays were performed in the presence of receptor-associated protein (RAP), which universally inhibits ligand binding to the LDL receptor family. Our data show that RAP eliminated platelet binding to immobilized FXI, but not to fibrinogen (Fig 2). Furthermore, the presence of soluble recombinant ApoER2' (sApoER2') abrogated platelet binding to FXI (Fig 2). Moreover, the presence of the LDL-binding domain 1 or 2 of ApoER2 reduced platelet adhesion to FXI by 78% and 55%, respectively (Fig. 2B).

In order to verify the ability of ApoER2 to mediate platelet binding to FXI, platelets from wild-type C57Bl/6 or ApoER2-deficient mice were incubated over surfaces coated with FXI or fibrinogen. As shown in Fig. 3, a greater than 90% reduction in platelet adhesion on FXI was observed for ApoER2-deficient platelets compared to wild-type (13.6 ± 1.4 vs. $1.2 \pm 0.5 \times 10^2$ platelets/mm^2 on FXI for wild-type vs. ApoER2$^{-/-}$, respectively). A similar level of reduction in binding was observed for wild-type platelets in the presence of binding domain 1 of ApoER2 (Fig. 3). The presence of soluble GPIbα (40 μg/ml) did not affect wild-type platelet binding to immobilized FXI (data not shown). Equivalent levels of adhesion were observed on fibrinogen for wild-type and ApoER2$^{-/-}$ platelets (155 ± 15 vs. $150 \pm 12 \times 10^2$ platelets/mm^2 on fibrinogen for wild-type vs. ApoER2$^{-/-}$, respectively).

Mechanisms of FXI-platelet interactions under flow

In order to evaluate the interaction between platelets and FXI in the presence of shear, washed human platelets were reconstituted with purified red blood cells (50% vol/vol, final platelet count 3×10^8 /ml) and perfused over immobilized FXI for 3 min at 600 s^{-1}. In the presence of shear, platelets in reconstituted blood formed aggregates on immobilized FXI (Fig. 4). Addition of the anti-GPIbα mAb, AK2, or LDL-binding domain 1 of ApoER2 blocked platelet adhesion to FXI under shear conditions (Fig. 4). Similar results were observed on FXIa surfaces in the presence of shear (Fig. 4).

Effects of Zn^{2+} and HK on platelet adhesion to FXI surfaces

FXI circulates in the plasma in complex with HK, and earlier studies have shown that FXI-platelet interactions in solution require both HK and the divalent cation, Zn^{2+}.[32, 33] As shown in Fig. 5A, FXI surfaces supported platelet adhesion in the presence of either vehicle or the combination of Zn^{2+} and HK. The presence of RAP abrogated platelet adhesion to FXI in the absence or presence of Zn^{2+} and HK (Fig. 5A). The degree of platelet adhesion to surfaces of the active site mutant FXI-Ala557, which lacks proteolytic activity, in the presence of vehicle was similar to adhesion to surfaces of native FXI (31.4 ± 4.2 vs. $33.9 \pm 3.6 \times 10^2$ platelets/mm^2 on FXI-Ala557 vs. native FXI, respectively).

FXIa surfaces supported platelet adhesion either in the presence of vehicle or the combination of Zn^{2+} and HK. Addition of RAP significantly, but incompletely, inhibited platelet adhesion to FXIa in the absence or presence of Zn^{2+} and HK (Fig. 5A). The remaining platelet adhesion on FXIa in each case was eliminated with the combination of RAP in combination with the serine protease inhibitor, D-phenylalanyl- L-prolyl-L-arginine chloromethyl ketone (PPACK, 40 μM).

Effects of known ApoER2 ligands on platelet adhesion to FXI

ApoER2 has been shown to bind to plasma components such as antiphospholipid antibody-dimerized beta 2 glycoprotein I (β_2GPI) and activated protein C (APC).[19, 22] As shown in Fig. 5B, platelet adhesion to FXI was unaffected by the presence of either plasma-derived or dimerized β_2GPI. Conversely, platelet adhesion to immobilized FXIa or APC was significantly reduced in the presence of dimeric β2GPI (Fig. 5B). Platelet adhesion to FXI or FXIa was not inhibited by the presence of APC in solution (data not shown).

Evaluation of soluble FXI binding to purified sApoER2'

To quantify direct binding of FXI to ApoER2, prescribed dilutions of FXI were incubated with immobilized sApoER2', and the level of bound FXI was determined using anti-FXI antibodies as described in "Materials and Methods". As shown in Fig. 6, soluble FXI bound to immobilized sApoER2' with an apparent affinity of 61 nM. The presence of RAP significantly reduced FXI binding to sApoER2' (Fig. 6). Taken together, our data identify ApoER2 as a novel receptor for FXI.

DISCUSSION

The aim of this study was to investigate the molecular mechanisms of FXI-platelet interactions. We identified FXI as a novel ligand for the receptor ApoER2. Our initial data demonstrated that purified, recombinant forms of ApoER2 abrogated platelet binding to FXI, and that platelets from ApoER2-deficient mice did not adhere to immobilized FXI. Immobilized ApoER2' specifically bound FXI in a cell-free assay with an affinity of 61 nM. Our data identify FXI as a ligand for the platelet receptor ApoER2.

FXI binding to platelets was first described over 30 years ago.[34] GPIbα was identified as the major receptor for FXI on activated platelets,[16, 17, 35] but studies have failed to show that GPIbα mediates FXIa binding to activated platelets under static conditions.[36, 37] The data reported here support a role for GPIbα in mediating shear-dependent interactions between resting platelets and both FXI and FXIa. The reason for the discrepancy between our results and earlier stucies is not clear, but it is noteworthy that ApoER2' has been shown to co-localize with the GPIb/IX/V complex on the platelet surface.[19] As FXI and FXIa exist as homodimers, perhaps one FXI/FXIa molecule can simultaneously bind both GPIbα and ApoER2' on the platelet surface. Since ApoER2 is expressed on the platelet surface at far lower levels than the GPIb complex,[19] this may explain why a total of only 1500 FXI binding sites per platelet have been reported,[33] even though GPIb is present at 25,000 copies per platelet.[38]

Since the expression of ApoER2 on platelets was discovered in 1999, platelet ApoER2 has been shown to bind a handful of plasma components.[19, 22, 39, 40] Data reported here suggest that FXI has a similar affinity for ApoER2 as recently-described ligands such as dimeric β_2GPI and activated protein C (APC).[22, 41] Autoantibodies to β_2GPI correlate highly with recurrent arterial thrombosis and fetal loss associated with antiphospholipid antibody syndrome (APS).[21] The *in vitro* properties of APS patient β_2GPI-anti-β_2GPI antibody complexes are mimicked by dimeric β_2GPI.[27] While neither dimeric β_2GPI nor APC in our experiments inhibited platelet adhesion to FXI, the presence of dimeric β_2GPI significantly reduced platelet adhesion to immobilized FXIa. β_2GPI has been shown to inhibit the activation of FXI by FXIIa and thrombin, whereas cleavage of β_2GPI at Lys^{317}-Lys^{318} by FXIa eliminates this inhibitory effect.[42] Because β_2GPI is able to interact directly with FXI and FXIa, and FXIa is able to cleave β_2GPI, further studies are required to determine the exact mechanisms leading to the differential effects of dimeric β_2GPI on platelet binding to FXI and FXIa.[42, 43] Perhaps the platelet receptor ApoER2 plays a role in mediating the interaction of β_2GPI with FXI on the surface of activated platelets and subsequent FXIa-mediated proteolytic cleavage of β_2GPI. It remains to be determined whether deregulation of this process by autoantibodies to β_2GPI may be an important mechanism for thrombosis in patients with APS.

While the universal LDL-receptor ligand-binding inhibitor RAP abolished platelet adhesion to FXI, its presence significantly, but incompletely inhibited platelet adhesion to FXIa. Previous studies have shown that the catalytic domain of FXIa is involved in platelet interactions.[44] In this study, the remaining platelet adhesion to FXIa was eliminated with the addition of the serine protease inhibitor, PPACK, in combination with RAP. A parallel set of observations was made in the presence of HK/Zn^{2+}. Taken together, these data point to a possible role for the catalytic domain in platelet-FXIa interactions, suggesting that a distinct, RAP-insensitive, site exists on the platelet surface that is capable of interacting with FXIa. One such possibility is that FXIa is capable of cleaving PAR receptors. Perhaps FXIa, once bound to the platelet surface via ApoER2, contributes to platelet activation via proteolytic cleavage of platelet PAR receptors. It remains to be determined if FXI binding to monocytes and endothelial cells, which express ApoER2,[22, 23] is mediated by ApoER2, and whether FXIa ligation of ApoER2 promotes a Reelin-like signaling pathway in these cells.

Recent findings by Tucker, et al. support the hypothesis that FXI activity is necessary for thrombus stability, as inhibition of FXI led to instability and dissolution of platelet-rich thrombi in a nonhuman primate thrombosis model.[45] FXIa bound to activated platelets has been shown to be resistant to inhibition by exogenous protease nexin 2.[36] It could be that ApoER2', in concert with GPIbα, plays a critical role in localizing FXI/FXIa to the growing platelet plug, subsequently facilitating FXIa activation of FIX, accelerating thrombin formation and thus promoting thrombus stability. Future work will be focused on identifying the significance of ApoER2 in FXI-dependent thrombus formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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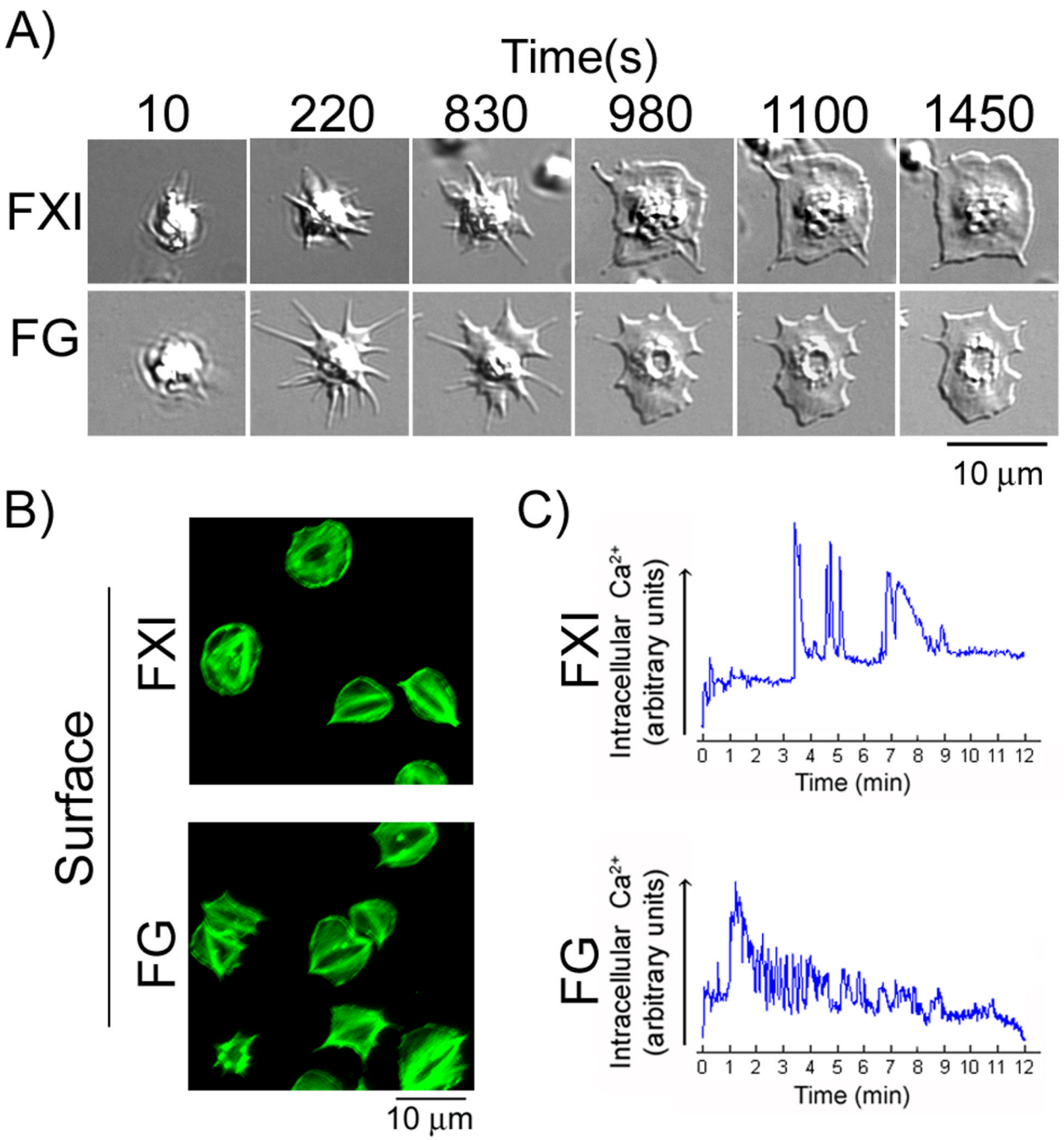

Figure 1. Platelet cytoskeletal reorganization on FXI surfaces

(A) Purified human platelets (2×10^7/ml) were exposed to surfaces coated with FXI or fibrinogen (FG) and observed in real time using differential interference contrast (DIC) microscopy. A representative time course of a single platelet spreading on each surface is shown. (B) Platelets adherent on each surface were fixed, permeabilized and stained for F-actin using FITC-conjugated phalloidin. (C) Purified human platelets loaded with the Ca^{2+}-sensitive dye Oregon Green BAPTA 1-AM were imaged as they made contact with FXI- (top) or FG-(bottom) coated surfaces. The graphs show the basal Ca^{2+} level of individual platelets upon arrival to the region of interest and the subsequent Ca^{2+} fluctuations over a 12 min period

of observation. The scale is in arbitrary units derived from the intensity of fluorescence emission.

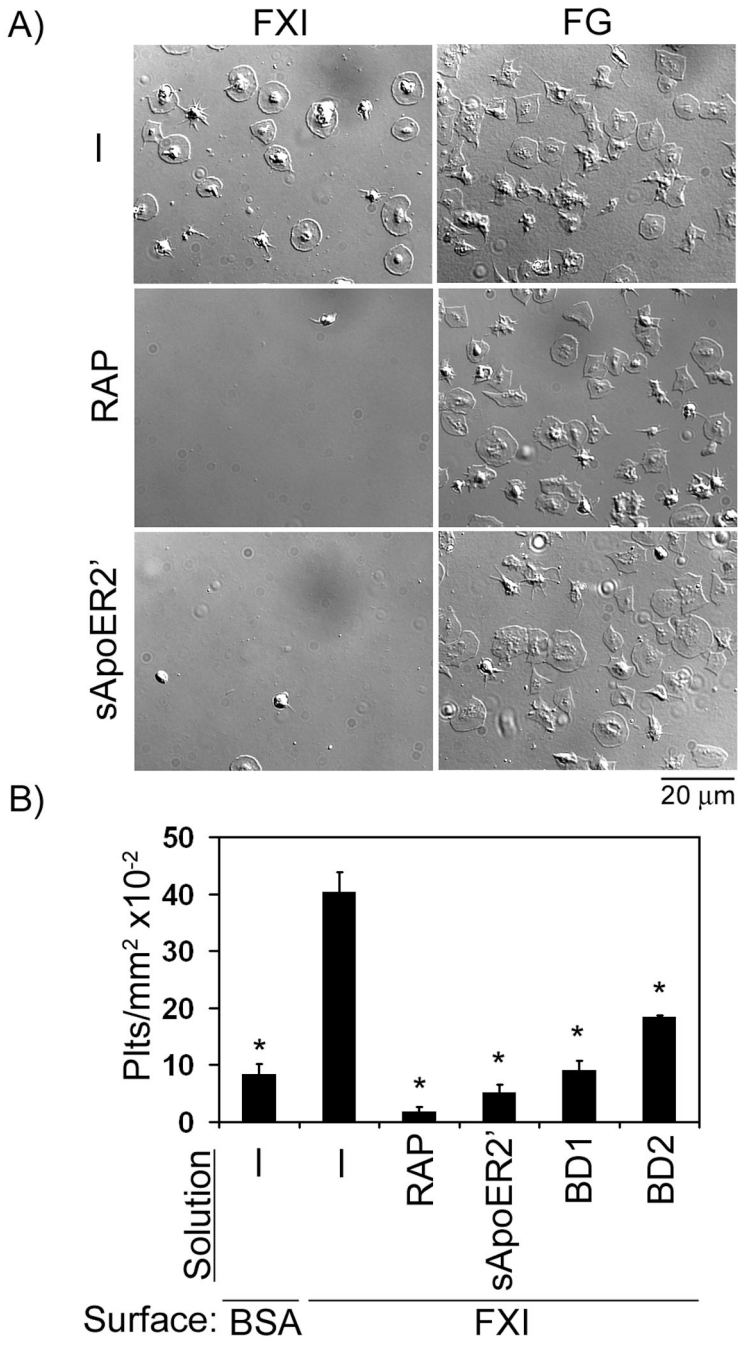

Figure 2. The role of ApoER2 in platelet adhesion to FXI

(A) Purified human platelets (2×10^7/ml) were pipetted onto surfaces coated with FXI or fibrinogen (FG) in the absence or presence of receptor-associated protein (RAP, 40 μg/ml) or soluble ApoER2' (sApoER2', 40 μg/ml), followed by incubation at 37°C for 45 min. (B) Additional adhesion experiments were performed on immobilized FXI in the presence of the LDL binding domains 1 or 2 of ApoER2 (BD1 or BD2, respectively, 40 μg/ml). Adherent platelets were analyzed for each suspension treatment and reported as adherent cells/$mm^2 \times 10^{-2}$. Values are reported as mean ± SEM of three experiments. *$P < 0.05$ compared to adhesion on FXI in the presence of vehicle.

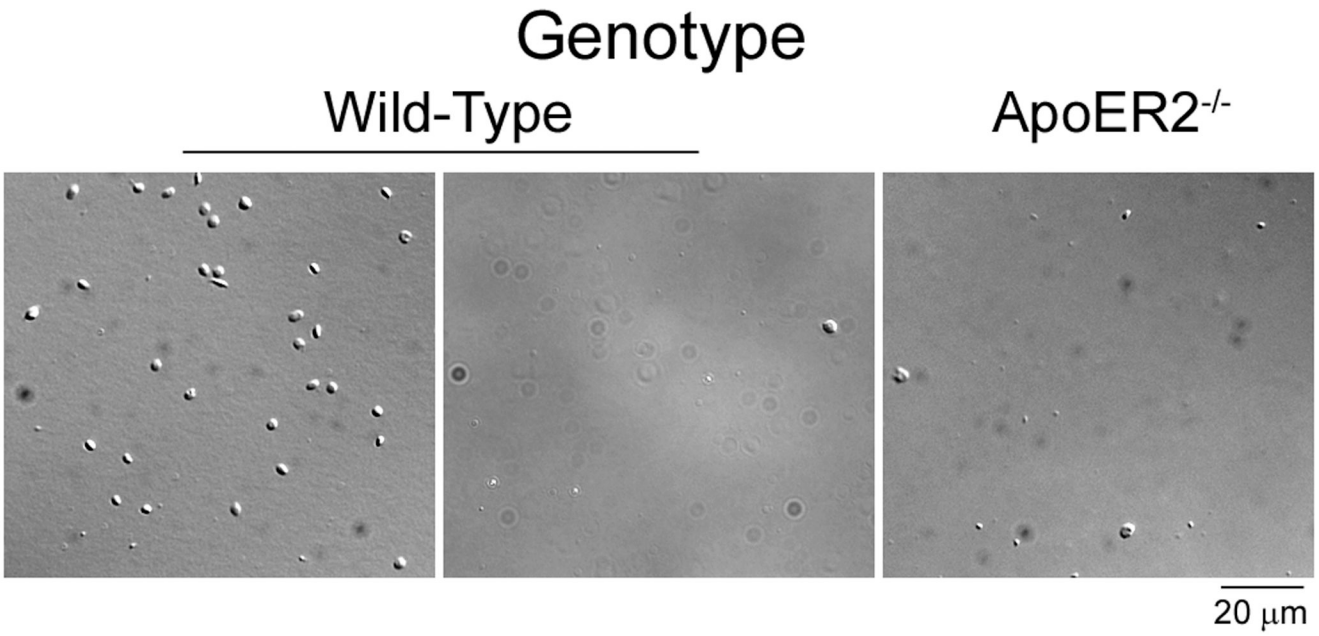

Figure 3. ApoER2-deficient platelets do not bind to FXI

Purified platelets (2×10^7/ml) from wild-type or ApoER2-deficient mice were incubated over immobilized FXI at 37°C for 45 min in the presence of vehicle (−) or the LDL binding domain 1 of ApoER2 (BD1, 40 μg/ml). Representative images from 3–4 experiments are shown.

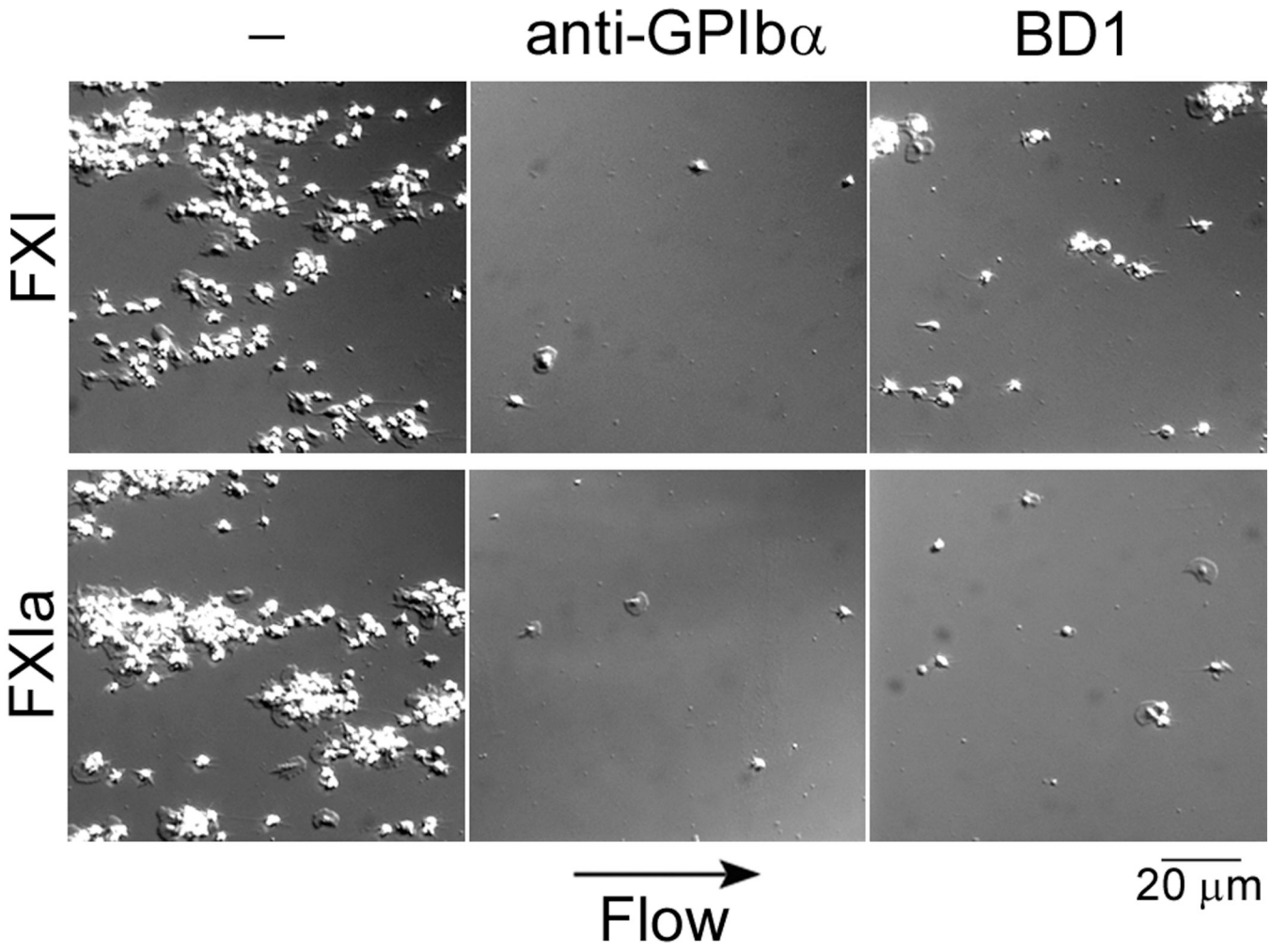

Fig. 4. Platelet binding to FXI under physiological shear

Human reconstituted blood (autologous packed red blood cells and washed human platelets combined to 50% vol/vol, yielding a final platelet concentration of 3×10^8/ml) was perfused over a surface of FXI or FXIa at a shear rate of 600 s^{-1} for 3 min in the presence of 25 μM Zn^{2+}. Separate experiments were performed in the presence of either an anti-GPIbα antibody (AK2, 20 μg/ml) or LDL-binding domain 1 of ApoER2 (BD1, 50 μg/ml). Images are representative of at least 3 separate experiments.

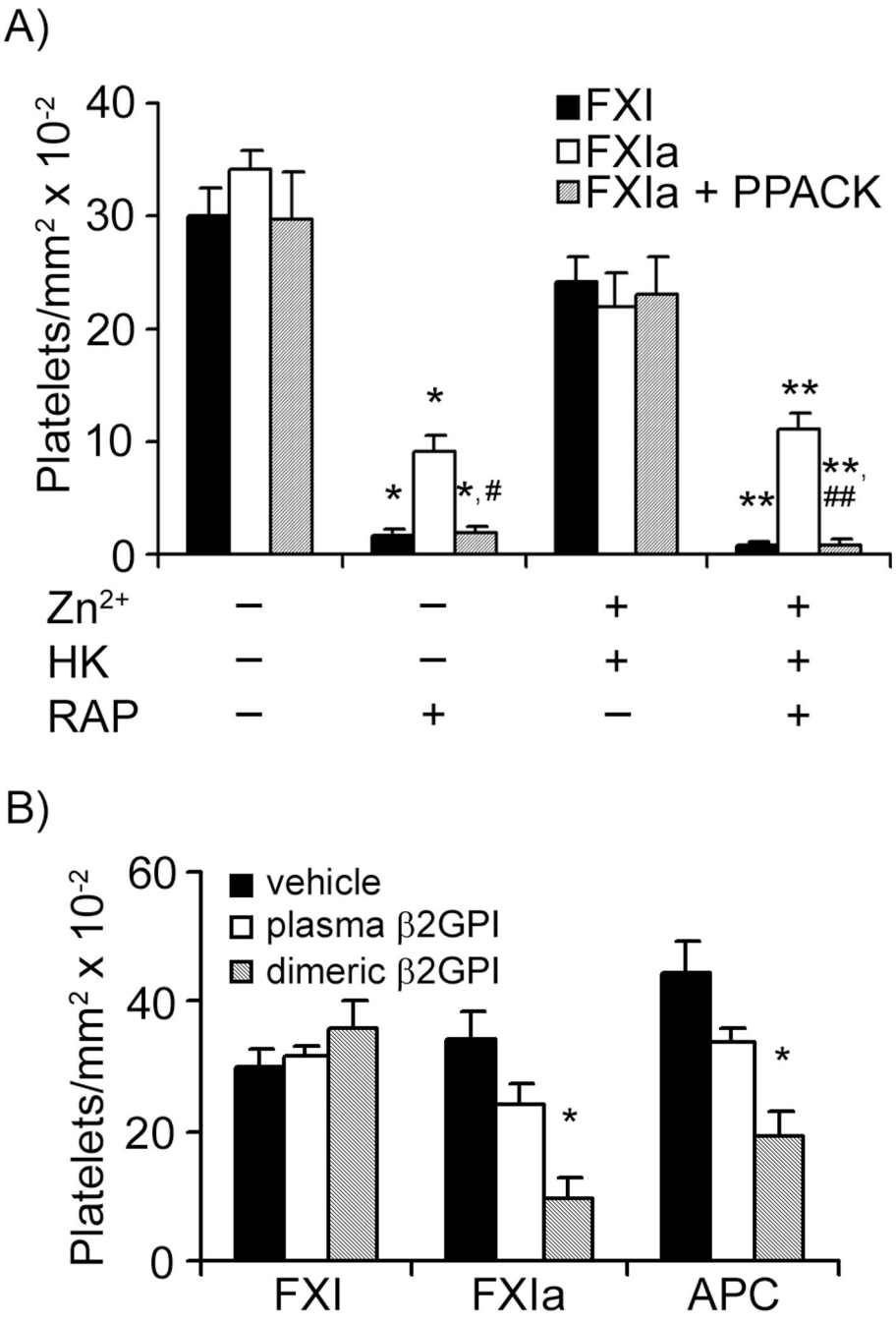

Figure 5. Platelet-FXI interactions in the presence of Zn^{2+}, high molecular weight kininogen or β2 glycoprotein I

Washed human platelets (2×10^7/ml) were incubated over surfaces of FXI or FXIa in the absence or presence of (A) Zn^{2+} (25μM) and high molecular weight kininogen (HK, 42 nM). Selected experiments were performed in the presence of PPACK (40 μM) or RAP (40 g/ml). $P < 0.05$ compared to adhesion on each respective surface in the presence of vehicle (*) or Zn^{2+} and HK (**). $P < 0.05$ compared to adhesion on FXIa with RAP in the presence of vehicle (#) or Zn^{2+} and HK (##). (B) Additional adhesion experiments were performed over FXI, FXIa or activated protein C (APC) in the presence or absence of plasma-derived beta2-glycoprotein I (β_2GPI, 350 nM) or dimeric β_2GPI (350 nM). Adherent platelets were calculated for each

treatment and are reported as adherent cells/$mm^2 \times 10^{-2}$. Adhesion numbers are reported as mean ± SEM of at least three experiments. *$P < 0.05$ compared to adhesion on each respective surface in the presence of vehicle.

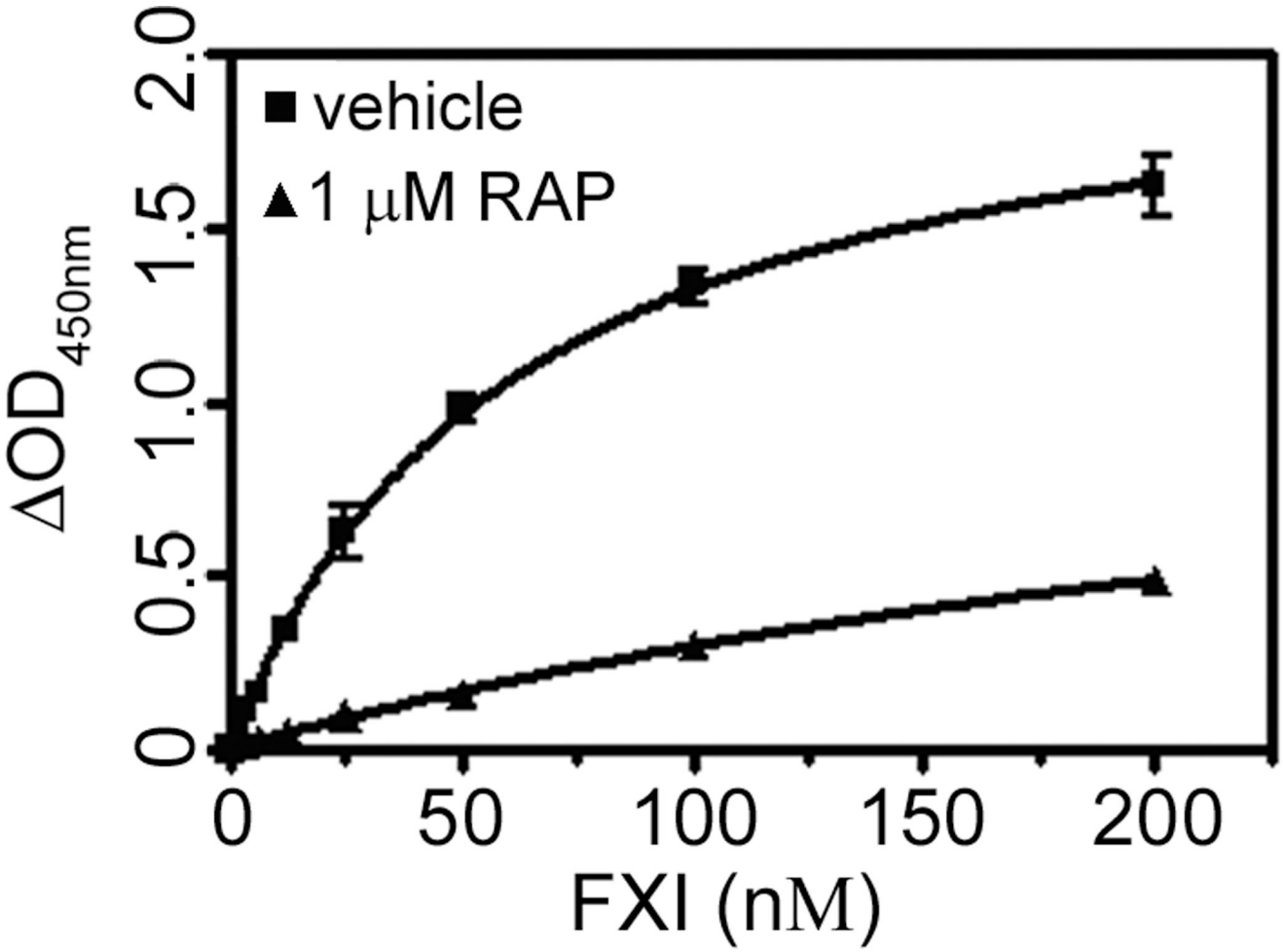

Fig. 6. Soluble FXI binding to purified ApoER2'

FXI at the indicated concentrations was incubated over a surface of recombinant ApoER2'. Designated experiments were performed in the presence of either vehicle (squares) or 1 μM RAP (triangles). Bound FXI was detected by anti-FXI antibodies as described in 'Materials & Methods'. Values are reported as mean ± SEM of 3 experiments.