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Internalization of Tissue Factor-Rich Microvesicles by Platelets Occurs Independently of GPIIb-IIIa, and Involves CD36 Receptor, Serotonin Transporter and Cytoskeletal Assembly

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ABSTRACT

Platelets are important in hemostasis, but also detect particles and pathogens in the circulation. Phagocytic and endocytic activities of platelets are widely recognized; however, receptors and mechanisms involved remain poorly understood. We previously demonstrated that platelets internalize and store phospholipid microvesicles enriched in human tissue factor (TF+MVs) and that platelet-associated TF enhances thrombus formation at sites of vascular damage. Here, we investigate the mechanisms implied in the interactions of TF+MVs with platelets and the effects of specific inhibitory strategies. Aggregometry and electron microscopy were used to assess platelet activation and TF+MVs uptake. Cytoskeletal assembly and activation of phosphoinositide 3-kinase (PI3K) and RhoA were analyzed by western blot and ELISA. Exposure of platelets to TF+MVs caused reversible platelet aggregation, actin polymerization and association of contractile proteins to the cytoskeleton being maximal at 1 min. The same kinetics were observed for activation of PI3K and translocation of RhoA to the cytoskeleton. Inhibitory strategies to block glycoprotein IIb-IIIa (GPIIb-IIIa), scavenger receptor CD36, serotonin transporter (SERT) and PI3K, fully prevented platelet aggregation by TF+MVs. Ultrastructural techniques revealed that uptake of TF+MVs was efficiently prevented by anti-CD36 and SERT inhibitor, but only moderately interfered by GPIIb-IIIa blockade. We conclude that internalization of TF+MVs by platelets occurs independently of receptors related to their main hemostatic function (GPIIb-IIIa), involves the scavenger receptor CD36, SERT and engages PI3-Kinase activation and cytoskeletal assembly. CD36 and SERT appear as potential therapeutic targets to interfere with the association of TF+MVs with platelets and possibly downregulate their prothrombotic phenotype. J. Cell. Biochem. 117: 448–457, 2016.

KEY WORDS: PLATELETS; TISSUE FACTOR; CELL-DERIVED MICROPARTICLES; CYTOSKELETON; INTERNALIZATION

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Platelets are known to participate in the pathogenesis of atherosclerosis promoting inflammatory conditions [Jackson, 2011; Lievens and von Hundelshausen, 2011]. Platelets also play a key role in the development of acute coronary syndromes and contribute to cerebrovascular events [Polgar et al., 2005; Davi and Patrono, 2007]. Exposure of TF at sites of disrupted atherosclerotic plaques is known to play an important role in the development of severe complications and potentially fatal conditions [Toschi et al., 1997]. Interactions between platelets and TF are unclear, but they are undoubtedly considered to be a critical component of atherothrombosis [Mackman, 2008; Cimmino et al., 2011]. Although it was classically accepted that vascular TF exposed on atherosclerotic lesions is the major contributor to thrombus formation and propagation [Hoffman and Monroe, 2003; Mackman, 2004], evidence from diffusion and kinetic models questioned the relevance of TF exposed on the vessel wall once covered by platelets [Hathcock and Nemerson, 2004]. It was hypothesized the existence of a circulating source of TF that would facilitate thrombus progression by transfer of new TF to the thrombotic interface [Giesen et al., 1999; Rauch et al., 2000]. The precise source of circulating tissue factor is still a matter of investigation, though different evidence points out that microparticles released from different cell lineages would contain active TF [Zillmann et al., 2001; Aras et al., 2004; Morel et al., 2004].

It has been proposed that platelets can carry variable amounts of TF under different experimental and clinical situations [Zillmann et al., 2001; Balasubramanian et al., 2002; Muller et al., 2003; del Conde et al., 2005; Perez-Pujol et al., 2005]. Whether platelet related TF is constitutionally present in platelets or transferred from other cells is subject of controversy, though the second possibility seems the more plausible [Camera et al., 2012]. TF associated with platelets is expressed upon in vitro platelet activation [Zillmann et al., 2001; Muller et al., 2003; Perez-Pujol et al., 2005]. In addition, there are reports on pathological conditions with increased cardiovascular risk showing augmented number of TF-positive platelets with an increased ability to generate thrombin, thus linking the platelet phenotype to the thrombotic complications in these diseases [Brambilla et al., 2008; Tilley et al., 2008]. Studies from our group applying flow cytometry and immunocytochemical techniques at ultrastuctural level demonstrated that platelets internalized human TF-rich microvesicles (TF+MVs) and that these MVs were primarily localized in vacuoles, trafficked through the OCS and eventually incorporated into alpha-granules [Lopez-Vilchez et al., 2007; Escolar et al., 2008].

Mechanisms involved in the transfer and association of TF+MVs with platelets must necessarily involve membrane receptors, cytoskeletal reorganization and activation of signaling pathways. GPIIb-IIIa is the main receptor for platelet adhesion and aggregation and is the target of the most powerful antiplatelet therapies [Topol et al., 1999]. CD36 is a type B *scavenger receptor* that recognizes cell-derived microparticles and contributes to thrombus formation [Ghosh et al., 2008]. Platelets possess 5-HT_{2A}—a G protein coupled receptor (GPCR) for serotonin—and a specific transporter (SERT) for this neurotransmitter. Previous evidence from our group indicate that specific blockade of the platelet SERT has an inhibitory action on the internalization of particles by platelets [Lopez-Vilchez et al., 2009]. Signals generated at the GPCRs results in activation of different members of the Rho family. Activation of RhoA has the potential to cooperatively activate PI3K and participate in the modulation of cytoskeletal rearrangement, endocytosis, vesicle traffic, and exocytosis of platelet granules [Heger and Collins, 2004].

The aim of the present study was to investigate the mechanisms involved in the association of TF+MVs with platelets. For this purpose, we investigated the influence of different inhibitory strategies targeting GPIIb-IIIa, CD36, and SERT platelet receptors. We also explored the contribution of cytoskeletal rearrangement and activation of RhoA and PI3K during the interactions of TF+MVs with platelets.

MATERIALS AND METHODS

DESIGN OF THE STUDY

Washed platelet suspensions were exposed to TF+MVs under stirring conditions as described in previous studies [Lopez-Vilchez et al., 2007; Escolar et al., 2008]. Platelet interactions with TF+MVs were assessed by optical aggregometry and ultrastructural techniques. Changes in cytoskeletal assembly and signaling proteins associated with cytoskeletal rearrangement and vesicle traffic (RhoA and PI3K) were also evaluated in platelets before or after exposure to TF+MVs. Inhibitory strategies with specific antibodies were applied to investigate the role of the major platelet glycoprotein GPIIb-IIIa or the CD36 *scavenger receptor* in the previous interactions. The effects of inhibitors of the SERT (escitalopram, SCIT), and PI3K pathways (wortmannin, Wo) were also explored. Activation with thrombin was used in some experiments as a positive control of strong and irreversible platelet activation and cytoskeleton polymerization.

Our investigations conform with the principles outlined in the Declaration of Helsinki. The study has been approved by our institutional Ethics Committee (ref/4700).

REAGENTS

Platelet agonists. Placental tissue factor (Thromborel® S; Siemens Healthcare, Marburg BmbH, Germany). Thrombin from human plasma (Sigma–Aldrich Chemie Gmbh (Steinheim, Germany).

List of inhibitory reagents. Abciximab (ReoPro®, clone 7E3; Lilly S.A., Madrid, Spain). Monoclonal mouse anti-human CD36 antibody (clone 185-1G2; LifeSpan BioSciences, Seattle, WA). Wo and SCIT oxalate were from Sigma–Aldrich Chemie Gmbh (Steinheim, Germany).

Reagents used for western-blot analyses. Coomassie Plus Bardford Assay™ Reagent (Thermo Scientific, Rockford, IL) for protein quantification in platelets lysates. Monoclonal rabbit anti-human phospho-PI3K p85(Tyr458)/p55(Tyr199) (Clone 19H8), anti-PI3K p85 and anti-human β-actin were from Cell Signaling Technology, Inc. Mouse anti-human RhoA antibody (ARH03; Cytoskeleton Inc., Denver, CO). Both goat anti-mouse and goat anti-rabbit IgG conjugated with HRP secondary antibodies were from Dako (Glostrup, Denmark). G-LISA™ RhoA Activation Assay Biochem Kit™ (Cytoskeleton, Inc., Denver, CO).

TISSUE FACTOR RICH-MICROVESICLES

TF+MVs from human placenta (Thromborel® S) were reconstituted according to the manufacturer's instructions to reach a final TF concentration equivalent to 1.1 nM. The TF concentration was calculated according to a previously described method for the evaluation of whole blood procoagulant tissue factor activity [Aras et al., 2004]. We previously reported that TF+MVs consists of microvesicles heterogeneous in size, ranging from 0.04 to 0.3 microns, that besides TF expose other concomitant antigens such as CD14, CD45, and CD62-P. Presence of TF in these microvesicles was previously characterized by immunocytochemistry and flow cytometry [Lopez-Vilchez et al., 2007].

BLOOD COLLECTION AND ISOLATION OF PLATELETS

Blood was obtained from healthy donors and collected into citrate/phosphate/dextrose at a final concentration of citrate of 19 mM. Platelets were separated as platelet-rich plasma (PRP) (120*g*) and washed 3-times with equal volumes of citrate/citric acid/dextrose (93 mM sodium citrate, 7 mM citric acid, and 140 mM dextrose), pH 6.5; containing 5 mM adenosine and 3 mM theophylline. The final pellet was resuspended at a concentration of 1.2×10^6 platelets/μL in a Hanks' balanced salt solution supplemented with dextrose (2.7 mM) and $NaHCO_3$ (4.1 mM), pH 7.2, and maintained for 50 min at 37°C before experiments were performed [Estebanell et al., 2000].

PLATELET AGGREGATION STUDIES

Aliquots of 400 μL of platelets suspensions were exposed to 44 μL of TF+MVs containing a final concentration of TF equivalent to 0.11 nM. The modifications in turbidimetric patterns during the aggregation of platelets were recorded for 5 min, in a four-channel Menarini PA 3210 Aggrecoder aggregometer (Menarini Diagnostic, Firenze), at 37°C and under continuous stirring. The results of these changes were expressed as percentage of maximum platelet aggregation. For sequential studies, interactions between platelets and TF+MVs preparations were stopped at different time points.

INHIBITORY STRATEGIES

In order to investigate the mechanisms implied in the uptake and traffic of TF+MVs, different inhibitory strategies were applied. The involvement of major glycoprotein GPIIb-IIIa was explored using Abciximab (Abcix, 20 μg/mL), an specific antibody to this glycoprotein. Implication of the *scavenger receptor* CD36 with internalization processes, was evaluated using a specific antibody (anti-CD36, 20 μg/mL). Activation of PI3K is required for cytoskeletal reorganization during endocityc and exocytic functions of platelets. PI3K mediated signaling pathways were blocked using Wo (1.6 μM). Based on previous evidence, we also explored the effect of the selective serotonin reuptake inhibitor SCIT (100 μg/mL). Washed platelet suspensions were incubated independently with the different inhibitors for 30 min at 37°C before exposure to TF+MVs.

ULTRASTRUCTURAL STUDIES

To confirm internalization of TF+MVs by platelets, platelet suspensions interacting with the TF+MVs, in the absence or presence of the inhibitory strategies, were processed for ultrastructural analysis with electron microscopy as previously described [Lopez-Vilchez et al., 2009]. Post-fixed pellets were dehydrated in ethanol, treated with propylene oxide and embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate to enhance contrast. Examination of grids was carried out in a Phillips (Mahwah, NJ) 301 electron microscope.

Activation of platelets and internalization of TF+MVs was semiquantitatively assessed in 25 to 30 platelets from different microscopic fields, for each of the different studied conditions. Activation of platelets was evaluated according to modifications in platelet shape, with those showing 6 or more pseudopodia per platelet being considered as platelets in advanced activation states (+++), those with 1 or 2 pseudopodia being considered minimally activated (+) and resting discoid platelets considered as non-activated (−). Internalization of TF+MVs was also semiquantitatively assessed, considering that platelet showing 5 or more vesicular structures in their cytoplasm in cross-sections were more actively interacting with MVs materials (+++) and those with one or two occasional vesicular structures being considered minimally interacting (+).

ANALYSIS OF CYTOSKELETAL POLYMERIZATION

Four hundred microliters of platelet suspensions at 1.2×10^6 platelets *per* microliter were exposed to 44 μL of either, TF+MVs or thrombin for different times. The polymerized cytoskeletal fraction was isolated by means of a lysis buffer containing Triton X-100, according to the procedure previously described [Escolar et al., 1993]. Triton-insoluble residues, corresponding to the polymerized cytoskeletal fraction, were recovered at 12,000*g* for 5 min at 4°C in an Eppendorf microfuge, and washed twice. The final pellet was solubilized with Laemmli's buffer (125 mM Tris-HCl, 2% (v/v) SDS, 5% (v/v) glycerol and 0.003% (w/v) bromophenol blue) containing 2 mM sodium ortho-vanadate and 5 mM N-ethylmaleimide, for 5 min at 90°C. The whole volume of solubilized proteins per condition was resolved by 8% SDS–PAGE and stained with Coomassie brilliant blue R-250 [Estebanell et al., 2000].

Digital images of the gels were captured using the ImageQuant LAS 500 GE imaging equipment (GE Healthcare Life Sciences AB, Sweden). Intensities of the bands in the gels were densitometrically analyzed using the ImageQuant TL v8.1 software (GE Healthcare Life Sciences AB) provided with this equipment. The association of each protein with the activated cytoskeleton was expressed as fold-increase over the amount of the same protein found in the respective lane corresponding to non-activated platelets. Furthermore, densitometry of each whole lane was measured as a global indicator of the polymerization of the cytoskeleton in each condition, and expressed also as fold-increase over the lane corresponding to resting platelets.

MODIFICATIONS IN PI3-KINASE SIGNALING

Platelets suspensions exposed to TF+MVs, in the absence and presence of the different inhibitory strategies, were lysed with Laemmli's buffer for 5 min at 90°C. Activation with thrombin was used in all the experiments as a positive control of strong platelet activation.

Protein concentration in each lysate was determined with Bradford protein assay [Bradford, 1976]. A hundred micrograms of solubilized proteins in the lysates were resolved by 8% SDS–PAGE

and transferred to a nitrocellulose membrane [Estebanell et al., 2000] for further identification of phospho-PI3K (p85) and PI3K (p85) with specific antibodies. The Intensity of the bands was densitometrically analyzed using the ImageQuant TL v8.1 software, and expressed as fold-increase over the amount of the same protein found in the respective lane corresponding to resting platelets.

ASSESSMENT OF RhoA ACTIVATION

Levels of RhoA activation in whole platelets lysates exposed to TF+MVs were determined with the RhoA G-LISA™ assay, which is based on the antibody sandwich principle. Interactions of platelets suspensions with TF+MVs were stopped at different times of incubation by addition of the lysis buffer provided with the assay. Protein concentration in each lysate was determined [Bradford, 1976] and further equalized between samples in the same experiment. Thrombin (0.1 U/mL) was used in all the experiments as a positive control of platelet activation and prolonged platelet cytoskeleton polymerization. The effects of the different inhibitory strategies were tested in platelets exposed to TF+MVs for 1 min, as the maximal intensity of the signals generated with TF+MVs usually occurs at 1 min [Lopez-Vilchez et al., 2007]. Absorbance was measured at 490 nm in a MultiSkan Ascent® (Thermo Electron Corporation, Finland). Results are expressed as nanograms of activated RhoA *per* miligrams of total protein.

In addition, association of RhoA with the detergent-resistant polymerized platelet cytoskeletons was investigated by western-blot in 12% SDS–PAGE, using a specific monoclonal antibody at 1/1000 dilution) followed by exposure to a secondary HRP-conjugated immunoglobulin at 1/2500 dilution), and revealed with chemiluminescence. The Intensity of the bands was densitometrically analyzed using the ImageQuant TL v8.1 software.

STATISTICAL ANALYSIS

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with raw data using the Student's *t*-test for paired samples and ANOVA. Minimal levels of statistical significance were established at $P < 0.05$.

RESULTS

EFFECTS OF INHIBITORY STRATEGIES ON PLATELET AGGREGATION DURING INTERACTIONS WITH TF+MVs

The activation of platelets with TF+MVs was measured by standard optical aggregometry. As shown in Figure 1, washed platelets exposed to TF+MVs, under stirring conditions, underwent reversible aggregation that was maximal at 1 min (44.1% ± 2.6%, n = 16), returning to basal levels after 5 min. The reversible platelet aggregation observed with TF+MVs completely differed to the irreversible platelet aggregation achieved when thrombin was used as reference agonist. Different inhibitory strategies aimed to prevent platelet activation were tested. Incubation of platelet suspensions with Abcix, anti-CD36, SCIT and Wo, prior exposure to TF+MVs, resulted in the prevention of the aggregating response (Fig. 1 and Table I).

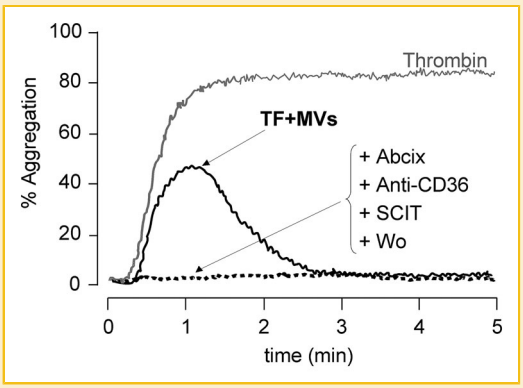

Fig. 1. Platelet aggregation induced by TF+MVs profiles and effects of inhibitory strategies. Addition of TF+MVs to washed platelet suspensions induced a reversible wave of aggregation with a maximal response at 1 min and returning to baseline values within 5 min. This pattern of response is completely different from the irreversible aggregation observed after activation with a reference concentration of thrombin. All the inhibitory strategies tested (Abcix, anti-CD36, SCIT, and Wo) prevented the reversible aggregation induced by TF+MVs.

INHIBITORY STRATEGIES DIFFERENTLY IMPAIR PLATELET INTERNALIZATION OF TF+MVs

Transmission electron microscopy was applied to evaluate ultrastructural changes in platelets exposed to TF+MVs after 1 min of interactions and patterns of vesicle uptake after 5 min of exposure to TF+MVs (Table I and Fig. 2). As shown in Figure 2, resting platelets display discoid shape and show a homogeneous distribution of intraplatelet granules, mitochondria and membranes of the OCS. In agreement with our previous findings [Lopez-Vilchez et al., 2007; Escolar et al., 2008], platelets exposed to TF+MVs for 1 min show signs of mild activation with loss of their discoid shape, partial release of the content of some granules and evidence of pseudopodia formation favouring interactions with other platelets. After 5 min of interactions with TF+MVs platelets have returned to a more quiescent stage, but microvesicles are recognizable within vacuoles and membranes of the OCS in the majority of platelets.

TABLE I. Activation of Platelets and Presence of TF+MVs Inside Platelets: Effects of Different Inhibitory Strategies

Inh. strategy	% Max. Aggreg.	Shape-change	Presence MVs
TF+MVs	44.1 ± 2.6%	+++	+++
+ Abcix	3.3 ± 0.7% *	++	++
+ anti-CD36	1.7 ± 0.4% *	++	+
+ SCIT	6.8 ± 1.9% *	+	+
+ Wo	1.3 ± 0.4% *	+	+

Quantitative evaluation of maximal platelet aggregation in isolated platelets exposed to TF+MV after 1 min of interaction. Qualitative evaluation of shape-change and presence of microvesicular structures in vacuoles or internal membranes of platelets evaluated at 5 min of exposure to TF+MVs.
Aggregometry results are expressed as percentage of maximal platelet aggregation achieved. TF+MVs alone (n = 16), Abcix (n = 13), anti-CD36 (n = 7), SCIT (n = 9), and Wo (n = 7). *$P < 0.01$ versus TF+MVs. Modifications in shape-change and presence of MVs were evaluated (from − to +++) in ultrastructural studies.

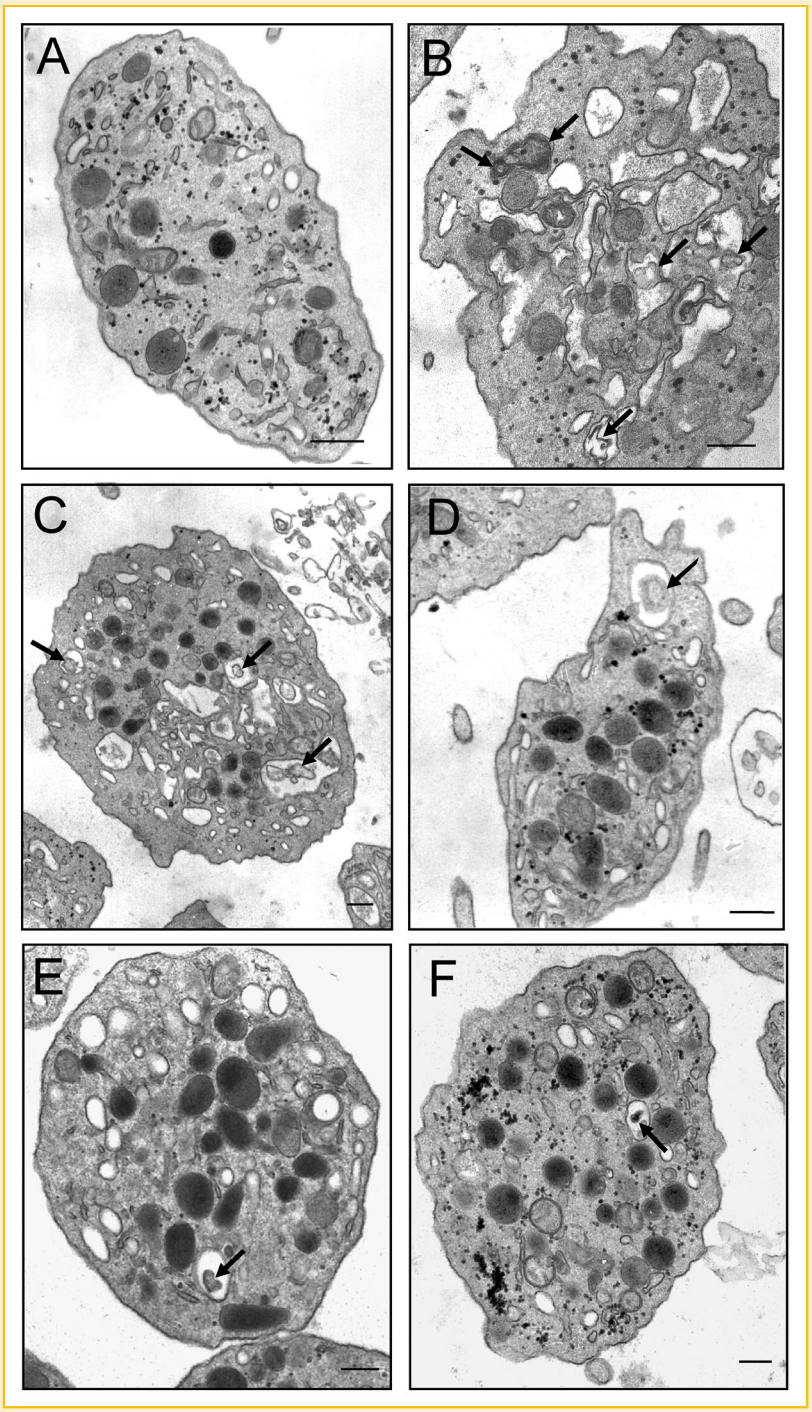

Fig. 2. Ultrastructural analysis of presence of TF+MVs in platelets and the effects of inhibitory strategies. (A) Resting platelets, with discoid shape and absence of MVs in the cytoplasm. (B–F) Representative micrographs of platelet vesicle uptake for each experimental condition after 5 min of exposure to TF+MVs. (B) In the absence of inhibitors, several internalized vesicles are visible inside membrane enclosed structures (arrows). (C) Inhibition of GPIIb-IIIa by Abcix did not fully prevent internalization of TF+MVs. In contrast, inhibitory strategies with (D) anti-CD36, (E) SCIT and (F) Wo, showed more efficient at interfering with the internalization of TF+MVs. Bar = 0.2 μm.

Inhibitory strategies with Abcix, anti-CD36, SCIT, and Wo prevented platelet–platelet interactions, thus confirming the results of aggregometry studies. Nevertheless, ultrastructural studies revealed that individual platelets may still show signs of a mild activation, such as loss of the discoid shape and dilated OCS. Interestingly, the efficacy of inhibitory strategies to prevent platelet aggregation did not fully correspond with their ability to interfere with internalization mechanisms. In this sense, anti-CD36, SCIT, and Wo seemed more efficient than Abcix at interfering with the internalization of TF+MVs (Table I and

Fig. 2). These findings indicate that blockade of platelet aggregation through a powerful inhibition of GPIIb-IIIa is not the more efficient strategy to prevent vesicle uptake. Indeed, SCIT and Wo were the most effective strategies at preventing ultrastructural signs of platelet activation and internalization of TF+MVs by platelets.

REORGANIZATION OF THE PLATELET CYTOSKELETON DURING INTERACTIONS WITH TF+MVs

Modifications in cytoskeletal assembly were evaluated in platelets before or after exposure to TF+MVs. As shown in Figure 3, interactions of TF+MVs with platelets promoted actin polymerization when compared to resting platelets (1.30% ± 0.04 fold-increase) and the association of the contractile proteins alpha-actinin (1.42 ± 0.08 fold-increase), actin binding protein (ABP) (1.65 ± 0.09 fold-increase), myosin (1.87 ± 0.10 fold-increase) and tropomyosin (1.52 ± 0.15 fold-increase) to the cytoskeleton after 1 min of exposure ($P < 0.05$). Modifications observed in platelets exposed to TF-MVs were comparable, with those after activation of platelets by thrombin at 5 min. However, these changes were not detected in samples of platelets exposed to TF+MVs for 5 min, where cytoskeletal proteins returned to resting conditions. Figure 3B summarizes the densitometric analysis of the different cytoskeletal proteins in the full lanes, and reflects overall modifications in the proportion of cytoskeletal polymerization throughout the different experimental conditions.

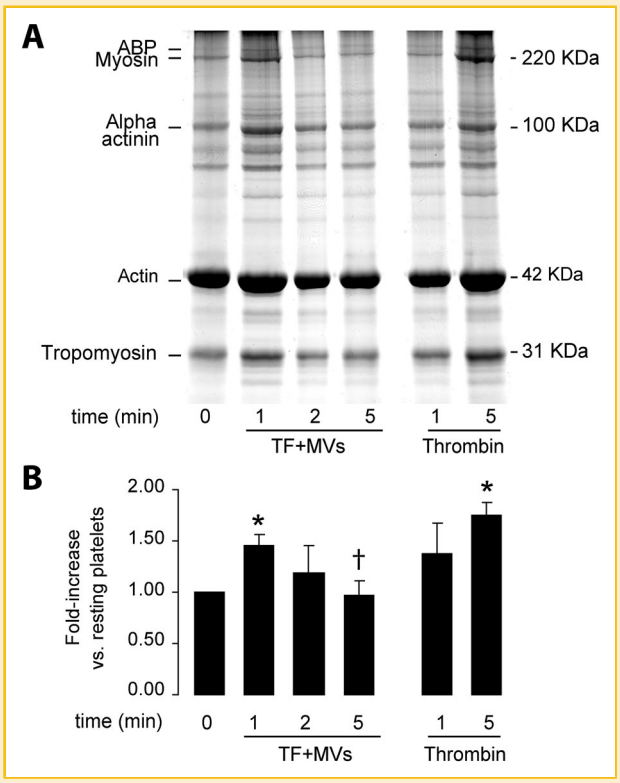

Fig. 3. Modifications in cytoskeletal assembly during the exposure of platelets to TF+MVs. (A) Coomassie Brilliant Blue–stained 8% SDS–PAGE gels showing protein profiles corresponding to the polymerized cytoskeletal fraction recovered in the detergent extracts from platelets suspensions. From left to right, lanes correspond to resting platelets (0 min), followed by platelets exposed to TF+MVs for 1, 2, and 5 min. Exposure of washed platelets suspensions to TF+MVs induced a moderate increase in actin polymerization and association of alpha-actinin, actin-binding protein (ABP), myosin, and tropomyosin with the polymerized cytoskeleton, being maximal at 1 min and returning to baseline levels after 5 min. The two last lanes illustrate the persistent modifications in the cytoskeletal assembly in platelets activated by thrombin at 1 and 5 min. (B) Bar diagrams summarize the densitometric analysis of the different cytoskeletal proteins in the full lanes expressed as fold-increase versus resting platelets. TF+MVs promote a transient increase in cytoskeletal assembly that is different from the consistent and irreversible assembly induced by thrombin. $^{*}P < 0.05$ versus resting samples (0 min); $^{\dagger}P < 0.05$ versus platelets exposed to TF+MVs for 1 min ($n = 4$).

ACTIVATION OF PI3K SIGNALING DURING THE INTERACTIONS OF TF+MVs WITH PLATELETS

As shown in Figure 4A, phosphorylation of PI3K followed in general the pattern of activation previously described for cytoskeletal reorganization. Exposure of platelets to TF+MVs triggered the phosphorylation of PI3K (p85) with a fold-increase over levels in the basal profile of 1.78 ± 0.14 at 1 min ($P < 0.01$) and returning to resting levels afterwards. Kinetics of phosphorylation of this protein in platelets activated by TF+MVs differ from that observed in platelets activated by thrombin where levels of phosphorylated protein remained consistently elevated after 2 min of activation with a fold-increase versus resting platelets of 2.06 ± 0.21 ($P < 0.01$).

PI3K activation was critically impaired by the different inhibitory strategies tested (Abcix, anti-CD36, SCIT, and Wo) as shown in Figure 4B. Densitometric analysis of bands confirm lower fold-increases versus resting platelets at 1 min of exposure to TF+MVs, with all the inhibitors.

ACTIVATION OF RhoA DURING INTERACTIONS OF TF+MVs WITH PLATELETS

The small GTPase RhoA participates in the modulation of cytoskeletal rearrangement, endo-exocytosis processes and intracellular vesicle traffic. Assessment of the small GTPase RhoA during the interactions of TF+MVs with platelets displayed maximal activation at 1 min of exposure to TF+MVs, progressively returning to resting levels within 5 min; opposite than observed in platelets activated by thrombin, where levels of RhoA activation increased and remained consistently elevated after 2 min of exposure to TF+MVs (Fig. 5A). Effects of inhibitory strategies on the activation of RhoA, after exposure to TF+MVs for 1 min, revealed different tendencies (Fig. 5B). Abcix, hardly affected the activation of RhoA, whereas the remaining inhibitory strategies (anti-CD36, SCIT, and Wo) showed downregulating effects on the activation of this small GTPase.

As shown in Figure 5C, RhoA became associated with the polymerizing cytoskeletal fraction, with maximal intensity in platelets exposed to TF+MVs for 1 min (5.8 ± 1.2 fold-increase vs. resting platelets; $P < 0.05$), decreasing gradually afterwards. This association was statistically prevented by all the inhibitory strategies tested (Fig. 5D).

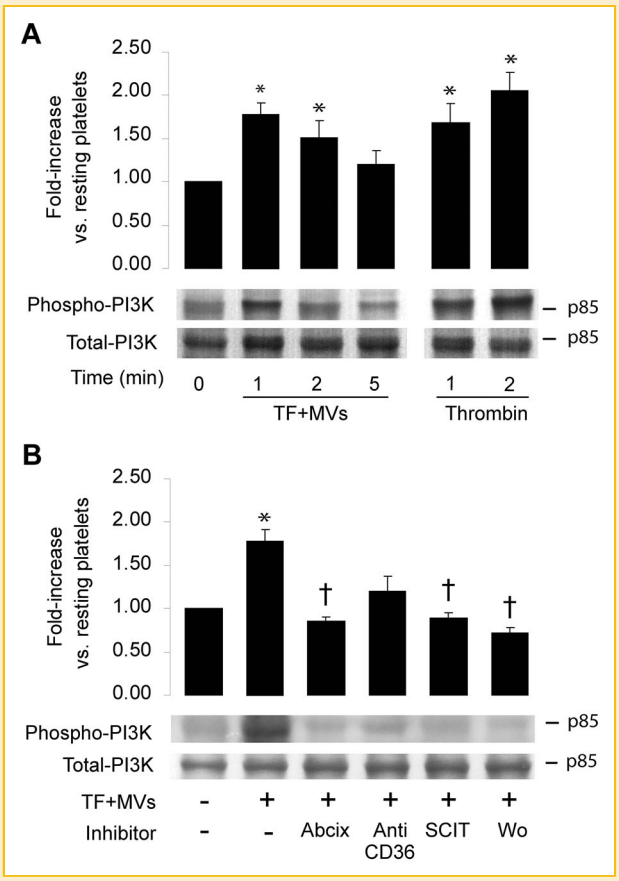

Fig. 4. Activation of PI3K during internalization of TF+MVs by platelets. Bar diagrams summarize the densitometric evaluation of the activation of PI3K (p85) as measured in blots from the different experimental conditions and expressed as ratio of increase versus resting platelets. (A) From left to right, lanes correspond to: resting platelets (0 min), platelets exposed to TF+MVs for 1, 2, and 5 min and platelets exposed to thrombin for 1 and 2 min. Activation of PI3K (p85) followed the kinetics observed for cytoskeletal assembly during TF+MVs interactions with platelets, with maximal levels at 1 min and returning to resting levels afterwards. Activation with thrombin for 1 and 2 min was used as positive controls of irreversible platelet activation showing a maximal phosphorylation of PI3K (p85) reaching maximum levels at 2 min. (B) Effects of the different inhibitory strategies on PI3K (p85) activation achieved at 1 min after exposure to TF+MVs. From left to right, lanes correspond to resting platelets or platelets exposed (+) to TF+MVs in the absence or presence of the different inhibitory strategies. All the inhibitory strategies (Abcix, anti-CD36, SCI, or Wo) caused important reductions on PI3K (p85) phosphorylations. $^{*}P < 0.01$ versus resting samples (0 min); $^{\dagger}P < 0.01$ versus platelets exposed to TF+MVs for 1 min ($n = 4$).

DISCUSSION

We previously demonstrated that platelets in suspension internalize TF+MVs, with vesicles becoming redistributed into vacuoles, trafficking through the OCS, and with the TF content eventually associating to the matrix of alpha-granules. In further studies, we provided in vitro evidence that platelet-associated TF contributed to an enhanced thrombus formation in studies with circulating through damaged vascular surfaces [Lopez-Vilchez et al., 2012]. Our present results demonstrate that transfer and traffic of TF+MVs to platelets does not involve GPIIb-IIIa, being more dependent on receptorial mechanisms related to CD36 and SERT. Furthermore, our present findings reveal that interactions of TF+MVs with platelets require cytoskeletal reorganization, with transient activation and translocation of RhoA to the polymerized cytoskeleton and concomitant activation of PI3K.

Results from our platelet aggregation studies confirm that exposure of washed platelet suspensions to TF+MVs induces a transient wave of reversible aggregation that was prevented with inhibitory strategies directed to block GPIIb-IIIa, CD36, SERT, or PI3K. Interestingly, our ultrastructural studies revealed that presence of TF+MVs within vacuoles and membranes of the platelet OCS were variably affected by the diverse inhibitory strategies tested. Strategies aimed at CD36, SERT, or PI3K showed more efficient at preventing incorporation of MVs into platelets than those directed to GPIIb-IIIa, such as Abcix. The antiplatelet agent Abcix, with a powerful inhibitory action on platelet aggregation mediated through GPIIb-IIIa, showed only a moderate ability to prevent association of TF+MVs with platelets. This indicates that inhibition of the major receptor GPIIb-IIIa, involved in platelet aggregation, does not necessarily prevent transfer of TF+MVs to platelets. In contrast, exposure of platelets to a specific inhibitor of the *scavenger receptor* CD36 resulted in a marked reduction in the presence of TF+MVs within platelets. We had previously reported that exposure of washed platelets to TF+MVs induced a significant expression of CD62-P and CD36, suggesting a role for CD36 in the internalization of TF+MVs by platelets [Lopez-Vilchez et al., 2009]. Ghosh et al. found that microparticles from human endothelial cells or monocytes associated with platelets [Ghosh et al., 2008]. This binding was not observed with platelets from CD36-negative donors and was inhibited by an anti-CD36 antibody. CD36 is known to trigger signaling mechanisms in phagocytes that result in internalization of bound ligands, such as oxidized low density lypoprotein or bacteria [Endemann et al., 1993; Stuart et al., 2005]. Our present findings on anti-CD36 strategies preventing the internalization of TF+MVs by platelets adds further evidence to the previous information and indirectly confirm the involvement of this receptor in the coverocytic function of platelets [White, 2005].

An important result from our studies was the effectiveness of the selective serotonin reuptake inhibitor (SSRI) SCIT to prevent the reversible aggregation induced by TF+MVs and more interestingly its ability to reduce their incorporation into platelets in ultrastructural studies. Serotonergic mechanisms have been associated with elevated cardiovascular risk [Escolar et al., 2005; Nakatani et al., 2005]. Previous investigations from our group indicated that serotonergic mechanisms potentiated platelet activation and favoured the internalization of TF+MVs and that these processes could have significant implications in thrombus formation and propagation [Lopez-Vilchez et al., 2009]. A previous report demonstrated that SSRIs may exert antithrombotic properties in both, experimental models and clinical settings [Sauer et al., 2003]. Further studies from our group demonstrated that the SSRI counteracted the potentiating effects of serotonin on platelet activation and thrombus formation [Galan et al., 2009], and that it could downregulate a prothrombotic platelet phenotype in patients

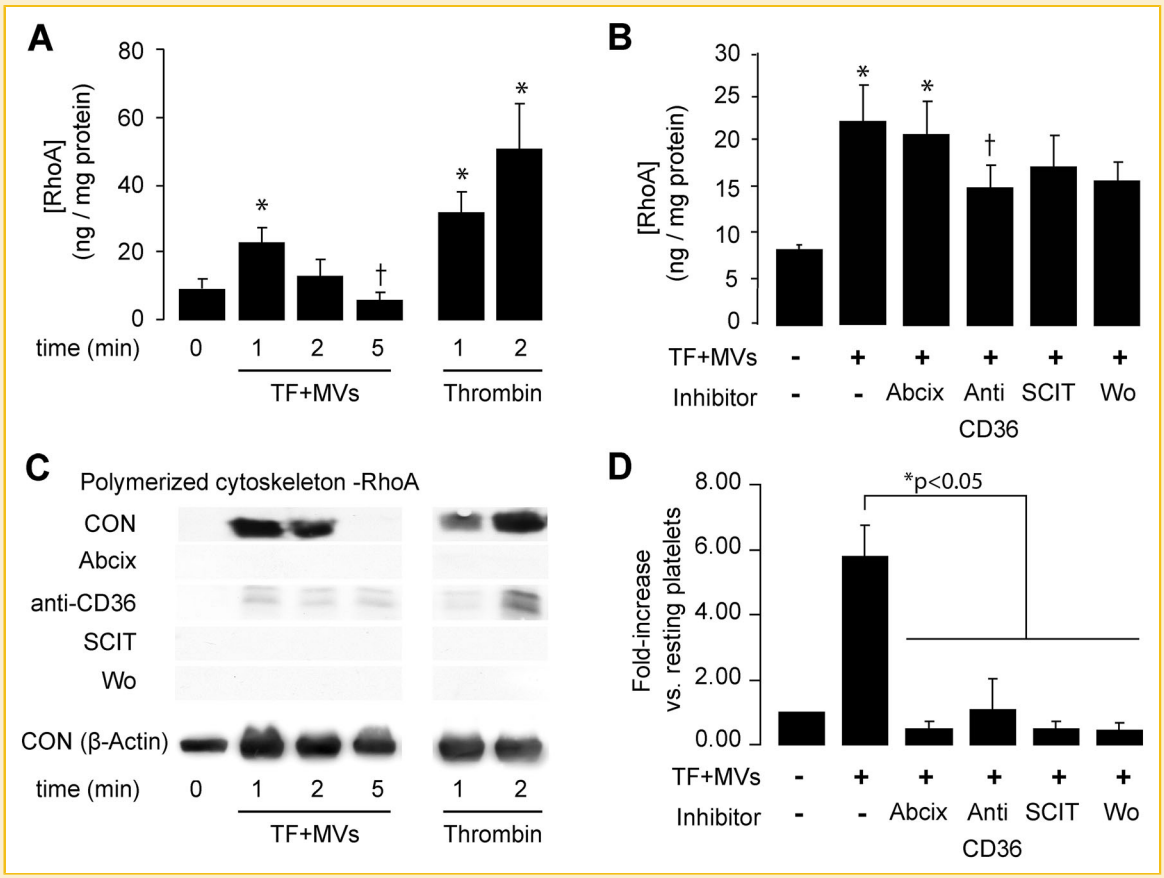

Fig. 5. Activation of RhoA and association with the cytoskeletal fraction during internalization of TF+MVs by platelets. (A) Bar diagram representing levels of RhoA activation in whole platelets lysates after exposure to TF+MVs for 1, 2, and 5 min. RhoA becomes activated reaching a maximum level at 1 min and gradually returning to resting levels thereafter. Activation with thrombin for 1 and 2 min was used as positive controls of irreversible platelet activation ($n = 4$, duplicates). (B) Bar diagram shows the effects of inhibitory strategies in the activation of RhoA during platelet interactions with TF+MVs, in resting platelets or platelets exposed for 1 min to TF+MVs, in the absence or presence of the different inhibitory strategies: Abcix, anti-CD36, SCIT, or Wo. Antibodies to CD36, SCIT, and Wo reduced the activation of RhoA, whereas Abcix showed no effects ($n = 4$, duplicates). (C) Association of RhoA with the polymerized cytoskeletal fraction recovered from the detergent extracts of platelets suspensions exposed to different experimental conditions. From left to right, lanes correspond to resting platelets (0 min), or exposed to TF+MVs for 1, 2, and 5 min. Platelets exposed to thrombin for 1 and 2 min were used as controls of irreversible platelet activation. RhoA associated to the polymerized cytoskeleton following a similar kinetics to the activation observed in whole platelets lysates, with the maximum labeling at 1 min after exposure to TF+MVs. Association of RhoA to the insoluble cytoskeleton was prevented by all inhibitory strategies. Labeling of β-actin was used as an indicator of cytoskeletal polymerization. (D) Bar diagram shows detailed densitometric quantification of bands corresponding to RhoA associated with the polymerized cytoskeleton, after 1 min of exposure to TF+MVs, in the absence or presence of inhibitory strategies ($n = 4$). $^{*}P < 0.05$ versus resting platelets (0 min); $^{\dagger}P < 0.05$ versus platelets exposed to TF+MVs for 1 min.

with major depression at the moment of diagnose [Galan et al., 2009; Lopez-Vilchez et al., 2014]. The reduction of intracellular serotonin concentration by specific inhibition of SERT is crucial for the activation of GTPases, that trigger platelet alpha-granules release and further potentiation of platelet aggregation [Walther et al., 2003]. Noteworthy of our studies with this antidepressant drug is that, as in the case of anti-CD36, SCIT was more efficient than Abcix at preventing the internalization of TF+MVs by platelets.

Cytoskeletal assembly plays a key role in shape change, spreading and platelet internal contraction, but is also necessary for particulate uptake and clearance [White, 2005]. Our present results confirm that cytoskeletal reorganization is involved in the capture and internalization of TF+MVs by platelets. Interactions of platelets with TF+MVs lead to the activation of PI3K and also of the small GTPase RhoA which translocated to the polymerizing cytoskeleton. PI3K regulates actin assembly necessary for platelet shape change, but also the activation of GPIIb-IIIa to promote stable aggregation [Jackson et al., 2004]. Inhibition of PI3K by Wo inhibited platelet shape change and reduced the incorporation of TF+MVs into platelets. These results are in agreement with those reported by Niu et al. [2012], who described how the PI3K inhibitor Wo blocked GPIIb-IIIa mediated activation of platelets. Interestingly, in our present studies we have also found a marked reduction in the internalization of TF+MVs. Rho GTPases are known to play an important role in the control of vesicular traffic regulating signaling pathways that link both extracellular and intracellular stimuli, with actin assembly and arrangement into the cytoskeleton [Bodie et al., 2001]. In our present study, we observed activation and association

of RhoA to the polymerized cytoskeleton during internalization of TF+MVs, both importantly impaired in the presence of the different inhibitory strategies, except for Abcix that did not prevent RhoA activation.

Whether TF associates with platelets is subject of an ongoing debate. Our previous in vitro studies demonstrated that washed platelets incorporate TF+MVs [Lopez-Vilchez et al., 2007] becoming more thrombogenic [Lopez-Vilchez et al., 2012]. Recent publications emphasize the scavenging, phagocytic, and immune-competent functions of platelets [Leslie, 2010; Morrell et al., 2014], abilities recognized several decades ago [Clawson and White, 1971; Zucker-Franklin, 1981]. These functions may in fact confirm the covercytic function of platelets, either spreading onto a large surface or internalizing it when smaller [White, 2005]. It would be difficult to understand how platelets that can internalize particulates, bacteria, nanoparticles, and viruses could specifically exclude circulating TF+MVs. Identification of receptors and signaling pathways involved in these processes should improve our knowledge on the mechanisms that contribute to the development of a prothrombotic phenotype in platelets exposed to inflammatory conditions.

Data provided by our present studies demonstrate that the specific blockade of GPIIb-IIIa was the least efficient strategy to prevent association of TF+MVs with platelets, whereas blockade of CD36 by a specific antibody inhibited to a greater extent these interactions. CD36 might be an attractive target for antithrombotic therapy, especially because no bleeding diathesis has been observed in humans or rodents with CD36 deficiency [Ghosh et al., 2008]. There is experimental and clinical evidence on the cardiovascular benefits from the use of a SSRI in both major depression and myocardial infarction patients [Sauer et al., 2003; Lopez-Vilchez et al., 2014]. Our present results may provide mechanisms for the beneficial action of SSRI in the previous clinical condition. In our studies, the PI3K inhibitor Wo was one of the most efficient inhibitory strategies tested. PI3Ks regulate a broad range of cellular responses, including cytoskeletal remodeling, and membrane trafficking. Inhibition of the PI3Kβ has also been shown to inhibit thrombus formation in different in vivo models without significantly affecting primary hemostasis [Sturgeon et al., 2008]. It is felt that current antiplatelet therapy with conventional agents may have reached the maximal efficacy in the prevention of ischemic cardiovascular events and associated complications [Diener, 2006]. There is a growing perception that newer antiplatelet drugs or associations with more powerful ones provide a marginally increased efficacy, which is frequently offset by more elevated bleeding rates. An important message of our studies is that interference with scavenging functions of platelets through inhibition of less canonical receptorial mechanisms (CD36 and SERT), or selective signaling pathways might open alternative strategies to overcome the limitations of current therapies.

In conclusion, interaction of TF+MVs with isolated platelets induces a moderate and rapidly reversible aggregation. Internalization of TF+MVs by platelets occurs independently of receptors related to their main hemostatic function such as GPIIb-IIIa, involves the *scavenger receptor* CD36, SERT and engages PI3K activation and cytoskeletal assembly. While it may be difficult to design specific strategies to prevent the activation of small GTPases, cytoskeletal arrangements or membrane fusion proteins, our results indicate that the CD36 *scavenger receptor* and SERT may become realistic therapeutic targets to downregulate a potential prothrombotic phenotype in platelets.

ADDENDUM

M. Diaz-Ricart, A.M. Galan, and G. Escolar conceived and designed the experiments. I. Lopez-Vilchez, M. Roque, C. Caballo, and P. Molina performed the experiments. All authors contributed to analyze and/or interpret the data. I. Lopez-Vilchez and A.M. Galan drafted the article. M. Diaz-Ricart, M. Roque, J.G. White, and G. Escolar revised critically the manuscript for important intellectual content. All authors read and approved the final version of the manuscript.

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