Escitalopram Impairs Thrombin-Induced Platelet Response, Cytoskeletal Assembly and Activation of Associated Signalling Pathways

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Abstract

Background  Serotonin reuptake inhibitors (SSRIs) may impair platelet function. Thrombin is a strong platelet agonist causing irreversible aggregation, release of granules’ contents, cytoskeletal rearrangement and activation of signalling pathways. We investigated the effects of the SSRI escitalopram (SCIT) on thrombin-induced platelet response.

Methods  Isolated platelets were exposed to SCIT and activated with thrombin. We evaluated (1) platelet response by aggregometry and flow cytometry; (2) modifications in cytoskeleton proteins and signalling pathways by electrophoresis and Western blot; and (3) ultrastructural changes in platelets by electron microscopy.

Results  SCIT inhibited platelet response to thrombin, measured as platelet aggregation and expression of activation markers CD62-P and CD63 from platelet granules. Platelet aggregation decreased in a dose-dependent manner, reaching statistical significance with SCIT ≥32 µg/mL (65.4 ± 6.8% vs. 77.7 ± 2.5% for controls; p < 0.05). Expression of activation markers was statistically reduced with SCIT ≥20 µg/mL (p < 0.05). SCIT impaired the polymerization of the actin cytoskeleton and association of contractile proteins during activation with thrombin (p < 0.05 with SCIT ≥50 µg/mL). Resting platelets incubated with SCIT became most spherical, with increased platelet roundness (p < 0.01, SCIT 50 µg/mL vs. control). SCIT interfered with signalling pathways modulated by thrombin (RhoA, PKC, Erk1/2 and PI3K/AKT).

Conclusions  Our data indicate that SCIT inhibits thrombin-induced platelet response and interferes with cytoskeletal assembly and related signalling pathways, thus resulting in compromised release of granules’ contents, reduced platelet activation and aggregation. These mechanisms may explain the antithrombotic benefits observed in patients treated with this SSRI, and could become new therapeutic targets for future antithrombotic strategies.

Keywords

► selective serotonin reuptake inhibitor
► thrombin
► release of granules
► cytoskeleton
► platelet function

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Introduction

Clinical evidence in the past two decades indicates a reciprocal relationship between cardiovascular risk and major depression, where platelets and an imbalance of serotonergic mechanisms seem to play a critical role.\(^1,2\) The high prevalence of depression in patients with coronary heart disease supports a strategy of increased awareness and screening for depression in this population.\(^3\) Platelet functionality involves multiple coordinated signalling pathways and the cytoskeleton assembly. A wide variety of receptors, glycoproteins, and transporters exist on the platelet membrane to modulate their haemostatic activity.\(^4\) Among these, platelets express the serotonin 5-HT\(_2\) receptor and the serotonin transporter (SERT), through which serotonin acts as platelet agonist or becomes rapidly incorporated into the platelet-dense granules. Serotonergic mechanisms in platelets are very similar to those found in serotonergic neurons. In this regard, biochemical similarities with neurons have led to consider platelets as surrogate neuronal models,\(^5,6\) always being conscious that they lack the connections and physiological structure of actual neurons. Nevertheless, platelets still prove useful in the study of some psychiatric diseases and their pharmacological treatment.\(^6\)

Selective serotonin reuptake inhibitors (SSRIs) are the most usual therapeutic approach to treat depressive disorders. Epidemiological studies indicate that patients with major depression treated with SSRIs have reduced cardiovascular risk compared with patients not receiving antidepressant therapy.\(^7-10\) SSRIs are designed to prevent rapid reuptake of serotonin at the intersynaptic cleft by blocking the SERT. However, SSRIs can interfere with the serotonergic mechanisms in vascular compartment, hence altering the haemostatic activity of platelets.

There is evidence of platelet hyperreactivity related to mood disorders.\(^6,11,12\) Previous studies from our group have demonstrated that serotonin induces prothrombotic phenotype in platelets,\(^13,14\) which can be corrected with the SSRI citalopram.\(^12,13\) Platelet's haemostatic activity is highly dependent on cytoskeletal organization and release of intracellular granules, mechanisms sharing elements with the secretory machinery of neurons.\(^15,16\) Thrombin is a strong platelet agonist, generated during the activation of the coagulation mechanisms, causing irreversible platelet aggregation through the differential activation of protease-activated receptors (PAR-1 and PAR-4)\(^17,18\) and through GPIb.\(^19\) Inhibition of thrombin generation is a common mechanism of current anticoagulant therapies.\(^20,21\)

The exact mechanisms by which SSRIs exert their antithrombotic action are not completely understood; whether it is a SERT-dependent process or through inhibition of second messengers is one of the questions that remains to be answered, though all evidence suggests interactions with multiple pathways. In the present study, we have investigated the effects of the SSRI escitalopram (SCIT) on thrombin-induced platelet response, cytoskeletal reorganization and modulation of signalling pathways.

Materials and Methods

Experimental Design

Our investigations conform to the principles outlined in the Declaration of Helsinki. This study has been approved by the Hospital Clinic Ethical Committee of Clinical Investigation (CEIC registry: 2013/86223). Healthy donors provided an informed written consent before the collection of blood samples.

The study was conducted on healthy volunteers with an in vitro experimental design. The effects of SCIT at 20, 30, 50, 75, 100, 150 and 200 \(\mu\)g/mL were tested on platelets exposed to thrombin; and the resulting changes in platelet reactivity, cytoskeletal assembly and signalling pathways were evaluated. Controls consisted of parallel samples not exposed to SCIT. Response of washed-platelet suspensions to thrombin (0.1 U/mL) was assessed by aggregometry and flow cytometry. Modifications in platelet morphology were evaluated by transmission electron microscopy. Finally, we used electrophoresis and Western blot to investigate activation of signalling pathways, downstream from thrombin receptors, involved in different aspects of platelet function such as shape change, cytoskeletal rearrangement, release of granules or stable platelet aggregation. Activation of the following specific signalling proteins in response to thrombin was explored: RhoA is a small GTPase involved in cytoskeletal arrangement and vesicle trafficking that translocates to the polymerized cytoskeleton. Conventional PKC plays an essential role in the secretion of platelet’s granular contents. Erk1/2 is also associated with release of granules and is essential for PAR-mediated TXA2 generation.\(^22\) And PI3K/Akt is related to integrin \(\alpha\)IIb\(\beta\)3 inside-out activation and regulates platelet aggregation.

Reagents

Platelet agonists and inhibitors: Thrombin from human plasma and SCIT oxalate were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

Reagents used for flow cytometry determinations: Paraformaldehyde solution (Electron Microscopy Sciences, Pennsylvania, United States); mouse anti-human CD41a conjugated with PerCP (BD Biosciences; California, United States); antibodies to human CD62-P (clone CLBThromb/6) and CD63 with PerCP (BD Biosciences; Milano, Italy); goat anti-human anti-PI3K p85, phospho-(Ser) PKC substrate (clone 19H8), rabbit anti-human anti-PI3K p85(Tyr458)/p55(Tyr199) (Rockford, Illinois, United States) for protein quantification in platelets lysates. Rabbit anti-human phospho-P3K p85(Tyr458)/p55(Tyr199) (clone 19H8), rabbit anti-human anti-P3K p85, phospho-(Ser) PKC substrate antibody and anti-human \(\beta\)-actin were from Cell Signaling Technology, Inc (Danvers, Massachusetts, United States). Mouse anti-human RhoA antibody (ARH03) was from Cytoskeleton Inc. (Denver, Colorado, United States). Both goat anti-mouse (#Z0420) and goat anti-rabbit (#P0448) IgG linked to horseradish peroxidase secondary antibodies were from Dako (Glostrup, Denmark).
Isolation of Platelets

Blood from healthy donors was collected into citrate/phosphate/dextrose (CPD) at a final citrate concentration of 19 mM. Platelets were first separated as platelet-rich plasma (PRP; 120 × g) and washed three 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 (1,100 × g). The final pellet was resuspended at the desired concentration in a Hanks’ balanced salt solution (136.8 mM NaCl, 5.3 mM KCl, 0.6 mM Na2HPO4, 0.4 mM KH2PO4, 0.2 mM NaH2PO4·2H2O) supplemented with dextrose (2.7 mM) and NaHCO3 (4.1 mM), pH 7.2, and maintained for 50 minutes at 37°C before experiments were performed.18

Platelet Aggregation Studies

Platelet suspensions at 0.4 × 10⁶ platelets/µL were incubated for 15 minutes at 37°C with different concentrations of SCIT. The aggregating response of platelets was assessed by using thrombin at 0.1 U/mL, a concentration that reflects the physiological response of platelets to thrombin offering a balanced contribution of the three different thrombin receptors to platelet activation (GPIb, PAR-1 and PAR-4).18,19 Modifications in turbidimetric patterns during the aggregation of platelets were continuously recorded for 2 minutes, in a four-channel Menarini PA 3210 Aggregometer (Menarini Diagnostic, Firenze), at 37°C and under continuous stirring. Results were expressed as percentage of maximum platelet aggregation.

Flow Cytometry Studies

Platelet activation with thrombin was assessed using flow cytometry, through the detection of CD62-P (P-selectin, an α-granule membrane protein) and CD63 (lysosomal integral membrane protein [LIMP]). These markers are exposed on the platelet membrane upon release of granules’ contents during activation with thrombin. Modifications in the expression of CD62-P and CD63 were evaluated in a Navios flow cytometer (Izasa–Beckman Coulter, Madrid, Spain). Platelet suspensions at 0.4 × 10⁶ platelets/µL incubated with different concentrations of SCIT and further activated with thrombin (0.1 U/mL) for 90 seconds were fixed with 0.3% paraformaldehyde for 15 minutes. Fixed platelets (5 µL) were added to polypropylene tubes preloaded with 50 µL PBS, pH 7.2, incubated with saturating concentrations of CD41a-PerCP and either CD62-P-FITC or CD63-FITC antibodies and diluted with 1 mL PBS for immediate analysis.13

Platelets were differentiated by their characteristic forward versus side scatter and by their positivity to CD41a. Histograms were composed from fluorescence data obtained in the logarithmic mode from 10,000 events analysed in each sample. For all studies, a negative control was performed with the matched fluorochrome. Acquisition files were analysed with the Kaluza Flow Cytometry Analysis software version 1.2.12286.11234 (Beckman Coulter, California, United States). Data were expressed as percentage of CD62-P and CD63-positive platelets. For this purpose, an analytical marker was set in the corresponding fluorescence channel to define a baseline of 2% in the resting platelet population. This marker was used as a threshold to determine the proportion of platelets exhibiting immunofluorescence above this level in all subsequent samples.

Electron Microscopy and Morphometric Analysis

Platelet suspensions incubated with SCIT with and without thrombin activation were processed for ultrastructural analysis as previously described.18 Briefly, platelets were mixed with an equal volume of 0.1% glutaraldehyde in White’s saline for 5 minutes and then were centrifuged. The supernatant was removed and replaced with 3% glutaraldehyde in the same buffer. Pellets were post-fixed with 1% osmium tetroxide in distilled water containing 15 mg/mL potassium ferrocyanide (pH 7.4) for 90 minutes at 4°C. After osmium fixation, samples were dehydrated in a graded series of ethanol concentrations, then treated with propylene oxide and embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate to enhance contrast and examined in a Phillips 301 electron microscope (Mahwah, New Jersey, United States).

Ultrastructural modifications in platelet shape of resting platelets were analysed using the Image J software (version 1.43m; Rasband, W.S., ImageJ; National Institutes of Health, Bethesda, Maryland, United States). Platelet outline was assessed in at least 25 of total platelets from different fields, for each different SCIT concentration studied (0, 20, 50, 100 and 200 µg/mL). Platelet roundness is automatically measured as 4 × area/π (major axis).2 Roundness ranges from 1 for a circle and approaches to 0 for very elongated objects. The area is calculated as π × (maximum radius)² and the major axis as two times the maximum radius.

Modifications in Platelet Cytoskeletal Reorganization

Cytoskeletal changes upon platelet activation with thrombin were assessed by measuring actin polymerization and association of contractile proteins α-actinin, actin binding protein, myosin and tropomyosin to the polymerized cytoskeleton. Platelet suspensions (400 µL) at 1.2 × 10⁶ platelets/µL were incubated with SCIT at different concentrations for 15 minutes, and subsequently exposed to thrombin (0.1 U/mL) for 3 minutes. The polymerized cytoskeletal fraction was isolated by means of a lysis buffer containing Triton X-100, according to a procedure previously described.19 Aliquots of platelets suspensions were treated for 30 minutes (at 4°C) with an equal volume of lysis buffer (100 mM Tris–HCl, pH 7.4, 2% Triton X-100) containing 10 mM ethylene glycol tetraacetic acid, 4 mM Ethylenedia- minetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidines, 2 mg/mL leupeptin, 2 mg/mL pepstatin A and 2 mM sodium orthovanadate, as protease and phosphatase inhibitors.

Triton-insoluble residues, corresponding to the polymerized cytoskeletal fraction, were recovered with an Eppendorf microfuge (12,000 × g, 4°C) and washed twice. The final pellet was solubilized with Laemmlli’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 orthovanadate
and 5 mM N-ethylmaleimide for 5 minutes at 90°C. The whole volume of solubilized proteins per each experimental condition was resolved by 8% SDS-PAGE and stained with Coomassie brilliant blue R-250.

**Western Blot Studies**

The signalling pathways involved in the effects of SCIT in thrombin-activated platelets were investigated by measuring the activation of the signalling proteins RhoA, protein kinase C (PKC), Erk1/2, phosphoinositide 3-kinase (p85-P13K) and Akt.

Association of small GTPase RhoA with the detergent-resistant polymerized platelet cytoskeletons was investigated by Western blot in 12% SDS-PAGE, using a specific monoclonal antibody followed by exposure to a secondary HRP-conjugated immunoglobulin, and revealed with chemiluminescence.

Activation of PKC, Erk1/2, p85-P13K and Akt was investigated in whole platelet lysates. Briefly, platelet suspensions at 1.2 × 10⁶ platelets/µL were incubated with SCIT for 15 minutes, and subsequently exposed to thrombin (0.1 U/µL) for 3 minutes. Platelets suspensions were lysed with Laemmlli’s buffer for 5 minutes at 90°C. Protein concentration in each lysate was determined with Bradford protein assay. Solubilized proteins (100 µg) in the lysates were resolved by 8% SDS-PAGE and transferred to a nitrocellulose membrane for further identification of signalling proteins with specific antibodies.

**Densitometric Analysis**

The intensity of the bands was densitometrically analysed using the Image J software (v1.43m; Rasband, W.S., ImageJ, National Institutes of Health) and expressed as fold-increase over the labeling intensity of the same protein/lane corresponding to nonactivated platelets. Profiles of cytoskeleton polymerization and phosphorylation of PKC substrates were globally analysed as the densitometry of the whole lane in each condition, and expressed also as relative fold-increase over the lane corresponding to resting platelets.

**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 using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, California, United States; www.graphpad.com). Minimal levels of statistical significance were established at p < 0.05.

**Results**

**SCIT Impairs Platelet Responses to Thrombin**

Platelet response to thrombin was reduced by SCIT (< Fig. 1A >). Maximal platelet aggregation in response to thrombin in the absence of SCIT was 77.7 ± 2.5% (n = 25). Exposure of platelet suspensions to SCIT inhibited thrombin-induced aggregation in a dose-dependent manner, with statistically significant reductions with SCIT at 32 µg/mL (65.4 ± 6.8%; p < 0.05 vs. control, n = 7). Platelet aggregation to thrombin was totally blocked with SCIT at concentrations ≥100 µg/mL (p < 0.001 vs. control, n = 15).

Expression of platelet activation markers CD62-P and CD63 was inhibited by SCIT. Results of flow cytometry studies are summarized in < Table 1 >. Adjusted baseline levels of platelets positive for either CD62-P or CD63 were 2.0 ± 0.01%. Activation of control platelets with thrombin resulted in statistically significant increase in the percentage of platelets expressing CD62-P and CD63 to 56.6 ± 7.3% and 34.7 ± 6.5%, respectively (p < 0.01 vs. control resting platelets). Expression of these activation markers was reduced in platelets previously incubated with SCIT at 20 µg/mL (p < 0.01 vs. control). Expression of CD62-P and CD63 was completely inhibited with SCIT at concentrations ≥75 µg/mL.

The ultrastructural analysis of platelets exposed to thrombin 0.1 U/mL revealed the classic activation signs of platelets affecting loss of discoid shape, pseudopod formation, internal contraction, and aggregate formation with reduced presence of α-granules in the cytoplasm (< Fig. 1B >). Incubation of platelets with increasing concentrations of SCIT prevented, to different extent, ultrastructural changes induced by thrombin (< Fig. 1C >), observing minimal evidence of platelet activation with the higher concentrations of SCIT (< Fig. 1D >).

**Modifications in Platelet Structure by SCIT**

Platelet cytoskeletal assembly in response to thrombin was altered by SCIT. Thrombin activation triggers polymerization of actin and association of contractile proteins α-actinin, actin binding protein, myosin and tropomyosin to the cytoskeleton. As summarized in < Fig. 2 >, these associations were significantly prevented at 100 µg/mL of SCIT and fully inhibited at 200 µg/mL. Moreover, we observed a statistical significant cytoskeletal depolymerization in resting platelets incubated with SCIT at 50 µg/mL (0.85 ± 0.05 fold-increase vs. control resting platelets; p < 0.05), which was more manifest with 200 µg/mL SCIT (0.71 ± 0.08 fold-increase vs. baseline resting levels in the absence of SCIT; p < 0.05).

Ultrastructural studies of resting platelets, incubated with different concentrations of SCIT, revealed that platelets had lost their discoid shape, turning more spherical (< Fig. 3A–D >). The morphometric analysis of platelet roundness (1 for a circle and 0 for maximal elongated objects) confirmed a progressive increment in platelet circularity upon incubation with SCIT (< Fig. 3E >). Control resting platelets displayed a roundness value of 0.47 ± 0.04. Incubation of platelet suspensions with SCIT at 50 µg/mL statistically increased this value to 0.59 ± 0.03 (p < 0.01 vs. control resting platelets). Platelet suspensions exposed to SCIT at 200 µg/mL resulted in further increased roundness values (0.81 ± 0.02; p < 0.01 vs. control platelets).

**Effects of SCIT on the Activation of Signalling Pathways in Platelets**

We investigated activation of signalling pathways involved in different facets of platelet function. RhoA is a small GTPase involved in cytoskeletal arrangement and vesicle trafficking that translocates to the polymerized cytoskeleton. Western
blot studies revealed increased association of RhoA to the cytoskeleton of platelet suspensions exposed to thrombin for 3 minutes. This association parallels the strong platelet activation and degree of cytoskeletal assembly. Incubation of platelets with increasing concentrations of SCIT prior to activation with thrombin resulted in lower RhoA presence, paralleling the degree of cytoskeletal polymerization, reaching statistical significance with SCIT 50 µg/mL (Fig. 4A).

Table 1  Platelet activation with thrombin is impaired by SCIT

<table>
<thead>
<tr>
<th>SCIT µg/mL</th>
<th>% Positive platelets</th>
<th>CD62-P (n = 5)</th>
<th>p-Value</th>
<th>CD63 (n = 10)</th>
<th>p-Value</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>56.6 ± 7.3</td>
<td></td>
<td></td>
<td>34.7 ± 6.5</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>32.8 ± 4.3</td>
<td>0.024</td>
<td></td>
<td>17.7 ± 3.9</td>
<td>0.038</td>
</tr>
<tr>
<td>50</td>
<td>10.5 ± 4.3</td>
<td>0.001</td>
<td></td>
<td>10.8 ± 1.9</td>
<td>0.003</td>
</tr>
<tr>
<td>75</td>
<td>4.9 ± 2.2</td>
<td>0.000</td>
<td></td>
<td>7.6 ± 1.7</td>
<td>0.001</td>
</tr>
<tr>
<td>100</td>
<td>2.6 ± 1.4</td>
<td>0.000</td>
<td></td>
<td>5.6 ± 1.5</td>
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</tr>
<tr>
<td>150</td>
<td>0.7 ± 0.4</td>
<td>0.000</td>
<td></td>
<td>5.4 ± 1.9</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Notes: Flow cytometry assessment of the activation marker CD62-P and the strong activation marker CD63 exposed on the surface of platelets activated with thrombin after incubation with different SCIT concentrations. Table summarizes the percentage of platelets expressing CD62-P and CD63 from an average baseline level of 2.0 ± 0.01%. Results are expressed as mean ± SEM; p-value summarizes paired t-test versus control platelets activated with thrombin.
Erk1/2 is involved in the release of granules and is essential for PAR-mediated TXA2 generation. Conventional PKC plays an essential role in the secretion of platelet's granular contents, and PI3K/Akt is related to integrin αIIbβ3 inside-out activation and regulates platelet aggregation. Activation of Erk1/2, PKC and PI3K/Akt in whole platelet lysates was augmented in response to thrombin; phosphorylation intensities, as fold-increases versus resting control platelets, were 5.0/C60.3 for Erk1/2, 2.1/C60.1 for PKC and 3.3/C60.3/1.8/C60.2 for PI3K/Akt, respectively (p < 0.05 vs. resting control platelets). Incubation with increasing doses of SCIT, prior to activation with thrombin, caused dose-dependent reductions in the intensities of signalling induced by thrombin, reaching statistical significance with SCIT at 50 µg/mL for Akt and Erk1/2, at 100 µg/mL for p85-PI3K and at 150 µg/mL for PKC. See - Fig. 4 for more detail.

Discussion

Clinical and epidemiological studies indicate that patients with major depression treated with SSRIs present lower cardiovascular complications in comparison to patients not receiving antidepressant therapy, suggesting a protective effect of antidepressant drugs in cardiovascular risk.7–10 Our present data demonstrate that SCIT impairs thrombin-induced platelet responses including platelet aggregation and release of granules. Moreover, our studies confirm that...
Fig. 4 Effects of SCIT on the activation of signalling pathways in platelets. Panels display representative band profiles for each signalling protein, accompanied with the corresponding bar diagram of the densitometric quantification expressed as fold-increase over the baseline in the absence of SCIT, and the correlation $R^2$ value. (A) Association of RhoA with the polymerized cytoskeletal fraction recovered from the detergent extracts of platelet suspensions exposed to different experimental conditions. From left to right, paired lanes comparing platelets at baseline and activated with thrombin (0.1 U/mL), for the different SCIT concentrations tested. RhoA associated with the polymerized cytoskeleton in thrombin-stimulated platelets; these associations were critically prevented by SCIT ($n = 3$). (B–E) From left to right, lanes correspond to resting platelets and thrombin-activated platelets with increasing concentrations of SCIT. Briefly, panels show activation of (B) Erk1/2, measured as phosphorylation of Erk1/2 ($n = 3$); (C) PKC, assessed through the resulting phosphorylation of PKC substrates on specific Serine residues ($n = 4$); (D) PI3K, investigated as phosphorylation of p85-PI3K ($n = 4$) and (E) Akt, explored as phosphorylation of Akt ($n = 3$). Western-blot studies revealed activation of Erk1/2, PKC, and PI3K/Akt in response to thrombin; incubation with increasing doses of SCIT, prior to activation with thrombin, caused dose-dependent reductions in the intensities of phosphorylation by thrombin. Bars are expressed as mean ± SEM; * $p < 0.05$ versus resting samples and # $p < 0.05$ versus thrombin-activated control platelets.
this antidepressant drug interferes with the cytoskeleton arrangement and signalling pathways. These findings could help explain the antithrombotic actions associated with SSRIs.

Thrombin is the strongest platelet agonist playing a major role in platelet thrombus growth and stability. Most of the current anticoagulant therapies are designed to decrease thrombin generation during activation of the coagulation mechanisms. Previous reports have demonstrated that SSRIs impair platelet aggregation to ADP and collagen agonists. However, the evidence of SSRIs’ role in thrombin-induced platelet activation is more controversial. Serebruany et al reported that incubation of PRP with the SSRI sertraline resulted in reduced platelet aggregation in response to collagen, ADP and thrombin.24 Using a different experimental approach, Carneiro et al25 revealed that genetic ablation of platelet 5-HT uptake in SERT+/− mice caused 50% reductions in platelet aggregation to thrombin. However, other in vitro studies using citalopram, a racemate containing the enantiomer escitalopram, reported impaired platelet aggregation to collagen and ADP, but not to thrombin.26–28 Our present study demonstrates that thrombin-induced platelet aggregation can be inhibited by SCIT in a dose-dependent manner.

Platelet haemostatic action implies shape change, pseudopodia emission, internal contraction and release of the granules’ contents.29 Tseng et al reported a direct inhibition with citalopram of TxA2 release in collagen-activated platelets.30 Similarly, other groups reported inhibitions by citalopram in the secretion of platelet’s granular contents, but using different experimental conditions.26,30,31 Our data indicate that SCIT consistently interferes with the release of platelet granule’s contents, showing statistical reductions in the expression of CD62-P and CD63 with the lowest concentration of SCIT tested (20 μg/mL). Reductions in the expression of granule’s contents would go along with the decreased platelet aggregating response observed in our study.

Correct cytoskeletal arrangement is critical for proper platelet morphology and function. Ultrastructural studies in the present study demonstrate that platelets incubated with SCIT lose their discoid shape and become more spherical. These alterations are accompanied by cytoskeletal depolymerization in resting platelets and reduced thrombin-induced cytoskeleton rearrangement in platelets incubated with SCIT. Piubelli et al, in a proteomic approach, that SCIT exerted an effect on proteins playing a role in cytoskeleton organization, neuronal development, vesicle-mediated transport and synaptic plasticity in a rat model of depression.32 Several reports suggest a link between SSRIs and calcium mobilization.33,34 Moreover, SSRIs have been described to intercalate in the plasmatic membrane due to their amphiphilic structure, like calcium antagonists, and even interfere with membrane receptors at higher (micromolar) concentrations.34–36 Calcium levels are crucial for correct cytoskeletal polymerization. EDTA is a strong calcium chelator widely used as anticoagulant. Platelets exposed to EDTA become spherical in shape,37 and they still show some responsiveness to thrombin, with impaired platelet aggregation, but preserved release of granules.38

Processes that occur upon stimulation of platelets with thrombin are mostly regulated by signalling pathways downstream G-protein–coupled receptors (GPCR). These result in feedback activation mechanisms with generation of thrombin, synthesis of thromboxane A2 and secretion of ADP, and the consequent stimulation of all major GPCRs.39 RhoA is a GTPase closely related to the platelet cytoskeleton, contributing to platelet’s shape change, Gα13-mediated GPCR crosstalk activation of integrin αIIbβ3, secretion of platelet’s granular contents and thrombus formation, among others.40 Stimulation of platelets with thrombin causes translocation of RhoA to the polymerized cytoskeleton. We reveal impaired translocation of RhoA to the platelet cytoskeleton in samples exposed to SCIT, paralleling the degree of cytoskeletal assembly in response to thrombin. Activation of RhoA with thrombin could in turn participate in the activation of PI3-kinase through direct interaction with the SH3 domain of the p85α subunit.41 In the present study, p85-PI3K becomes activated with thrombin in a similar proportion than RhoA; however, reductions by SCIT followed different kinetics. The PI3K/Akt signalling pathway is involved in the inside-out activation of integrin αIIbβ3 and regulates platelet aggregation; statistical inhibition of these proteins matches the impaired platelet aggregation previously measured in our studies.

As stated earlier, we also found that SCIT remarkably impairs the release of platelet granules. Conventional PKC plays an essential role in the secretion of platelet granules’ contents, and Erk1/2 is also involved in the release of granules and is essential for PAR-mediated TXA2 generation.42 Platelet Erk2 activation by thrombin is dependent on calcium and conventional protein kinases C.43 Our present data confirm thrombin-induced activations of PKC and Erk1/2 and demonstrate dose-dependent inhibitions with SCIT. Changes by SCIT in the activation of signalling pathways involved in platelet function, downstream from thrombin receptors, demonstrate a clear involvement of SSRIs in platelet signalling. A study by Tseng et al investigated activation of signalling pathways in response to ADP, including Akt and PKCα, and assessed their inhibition with SSRI; however, they suggest that SSRIs may influence the signalling pathway upstream from the activation of Syk in platelets.44

Taking our results altogether, and considering the effects of SCIT in the different critical aspects of platelet functionalism, we should expect cross interactions between SSRIs and other biological pathways. Serotonin receptor 5H12A, PARS and adrenergic receptors belong to the family of GPCRs sharing some signalling pathways. The α-adrenergic component of the sympathetic nervous system plays a major role in the pathophysiology of major depression. Treatment of major depression patients with SSRIs cause reductions in their sympathetic nervous activity in a manner likely to reduce cardiac risk.43,44 On the other side, there is concern regarding the occurrence of adverse bleeding events associated with the use of SSRI. Several studies have reported that SSRIs interfere with platelet function, increasing bleeding
risk when used alone or in combination with antiplatelet therapies.\textsuperscript{45,46} Present guidelines for the secondary prevention of cardiovascular events contemplate the indication for dual antiplatelet therapy (DAPT) associating the use of aspirin with clopidogrel, prasugrel or ticagrelor. It is evident that DAPT enhances the risk of bleeding; however, the benefit/risk balance seems to be in favour of these associations. Clinical experience indicates that adults with atherosclerotic cardiovascular disease and co-existent anxiety or depression are more likely to use a combination of SSRI and platelet aggregation inhibitors.\textsuperscript{47} Thus, despite the potential bleeding risk, the association of SSRI with antiplatelet agents may be acceptable in the clinical practice, most likely because the expected bleeding risk would be minor to moderate.

Platelet functionality depends on coordinated signalling pathways and correct reorganization of cytoskeletal components. Our data indicate that at elevated concentrations, SCIT inhibits thrombin-induced platelet response and interferes with the cytoskeletal assembly, thus resulting in compromised release of granules’ contents, reduced platelet activation and aggregation. These findings could explain the antithrombotic benefits observed in patients treated with SCIT. Our present results demonstrate a clear inhibitory action of SCIT on strong signalling pathways, and also suggest new therapeutic targets for future antithrombotic strategies. Whether different SSRIs would exert similar antiplatelet actions should be specifically investigated.

## What does this paper add

- Thrombin is a strong platelet agonist. Modulation of thrombin-induced platelet response is important to prevent thrombotic complications.
- Serotonergic mechanisms are involved in haemostasis. Serotonergic mechanisms in platelets share similarities with those in serotonergic neurons.
- Patients with major depression treated with SSRIs have reduced cardiovascular risk, compared with patients not receiving antidepressant therapy.

## What is known about this topic

- Our data demonstrate that the SSRI escitalopram (SCIT) inhibits thrombin-induced platelet response and interferes with cytoskeletal arrangement.
- The ultrastructural findings show that platelets incubated with SCIT lose their discoid shape and become more spherical.
- Our results reveal a clear inhibitory action of SCIT on strong signalling pathways, and also suggest a new therapeutic antithrombotic strategy.

## Conflict of Interest

Authors have no conflict of interest to declare.

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