

Platelets and Blood Cells

Serotonergic mechanisms enhance platelet-mediated thrombogenicity

Ana M. Galan¹; Irene Lopez-Vilchez¹; Maribel Diaz-Ricart¹; Fulgencio Navalon¹; Esther Gomez²; Cristobal Gasto²; Gines Escolar¹

¹Servicio Hemoterapia-Hemostasia, Hospital Clinic, CDB, IDIBAPS, UB, Barcelona, Spain; ²Instituto Clínic de Psiquiatria, Hospital Clinic, ICN, Barcelona, Spain

Summary

Although it is generally acknowledged that serotonin (5-HT) is a weak agonist for human platelets, recent information suggests an association between serotonergic mechanisms and cardiovascular risk. We investigated the action of 5-HT on adhesive, cohesive and procoagulant properties of human platelets. Impact of 5-HT on whole blood coagulation and thrombin generation was measured by modified thromboelastometry (TEM) and specific fluorogenic assays. We evaluated the effects of 5-HT on thrombus formation in an in-vitro model of thrombosis using human flowing blood. In platelet-rich plasma (PRP), 5-HT favoured the expression of CD62-P, and procoagulant molecules on platelet membranes. These effects were potentiated in the presence of Ca⁺⁺ and/or ADP. Incubation with 5-HT accelerated clotting times and augmented clot strength in whole blood TEM, and enhanced thrombin generation in PRP. In perfusion studies, 5-HT

significantly increased fibrin deposition at low shear (300s⁻¹) and enhanced platelet thrombus formation on the damaged vascular surface at high shear (1,200s⁻¹). Selective inhibition of serotonin reuptake (SSRI) attenuated effects of 5-HT on platelet activation and downregulated the prothrombotic tendencies observed in the previous experimental conditions. In general, reductions of thrombogenic patterns observed with SSRI were more evident under shear conditions (aggregation and perfusion systems) and less evident under steady conditions (TEM and thrombin generation assays). In conclusion, 5-HT is not a weak agonist for human platelets; instead it accentuates platelet activation, potentiates procoagulant responses on human blood and increases thrombogenesis on damaged vascular surfaces. The remarkable antithrombotic actions achieved through SSRI deserve further mechanistic and clinical investigations.

Keywords

Platelet physiology, platelet pharmacology, serotonin, thrombogenesis

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Introduction

5 Hydroxytryptamine (5-HT) is an abundant component of platelet-dense bodies (1). *In vitro*, 5-HT is rapidly incorporated into platelets, stored in the dense bodies and secreted during platelet activation (2). Once released, 5-HT has two known roles: vasoconstriction and platelet activation. Platelets possess receptors for 5-HT (5-HT_{2A}, 5-HT₃) (3, 4), and a 5-HT transporter (SERT) in their membranes (5). Despite the ubiquitous distribution of 5-HT in platelets and plasma, the presence of several receptors in platelets, and its pleiotropic pharmacological effects (6), the functional implications of serotonergic mechanisms have not

been considered of relevance for haemostasis. No haemorrhagic disorders have been associated with quantitative or qualitative alterations of 5-HT or its receptors. Standard aggregometry studies indicate that 5-HT is an apparently weak platelet agonist with a certain ability to potentiate the aggregating response of other activating agents (7, 8).

Recent studies are opening a debate on the initial perception of 5HT as a feeble contributor to haemostasis. Different lines of evidence have established a link between alterations of serotonergic mechanisms in mood disorders and their possible relation to the development of an enhanced cardiovascular risk (9, 10). A series of clinical studies suggest that depression could be an in-

Correspondence to:

Ana M. Galán
Servicio de Hemoterapia-Hemostasia, Hospital Clinic
Villarroel 170, 08036 Barcelona, Spain
Tel.: +34 93 227 54 00 Ext.: 2034, Fax: +34 93 227 93 69
E-mail: agalan@clinic.ub.es

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dependent risk factor for cardiac events (11). Several studies have evidenced a link between increased risk of cardiovascular complications in clinically depressed patients (12) and a possible reduction of this risk after treatment with selective serotonin reuptake inhibitors (SSRI) (13–15).

Taking into consideration the previous statements and the link between mood disorders and cardiovascular risks, the present study was designed to evaluate at experimental level the possible implications of serotonergic mechanisms in haemostasis. With this purpose in mind we evaluated effects of 5-HT on adhesive, cohesive and procoagulant function of platelets under several experimental situations including thrombogenesis models with circulating blood. Standard aggregometry, flow cytometry, thromboelastography and thrombin generation assays were used. Modifications of platelet adhesion and thrombus formation in a well established in-vitro model of thrombosis with collagen rich vascular surfaces exposed to flowing human blood at different shear rates. The in-vitro effects a selective serotonin reuptake inhibitor was evaluated under all the previous experimental conditions.

Materials and methods

Reagents and antibodies

Whole blood was anticoagulated with low molecular weight heparin (LMWH, Fragmin[®], Pharmacia, Madrid, Spain) at a final concentration of 20 U/ml, or with Citrate/Phosphate/Dextrose Buffer (CPD) to a final concentration of citrate of 19 mM. Serotonin hydrochloride was from Fluka (Sigma-Aldrich Switzerland, Buchs SG, Switzerland). Citalopram (Seropram[®]) was from Lundbeck (Opfikon-Glattbrugg, Switzerland). Aggregpack ADP and Collagen reagents were from ArkRay Inc (Kyoto, Japan). Arachidonic Acid was from Helena Biosciences Europe (Sunderland, UK). Platelets were analysed by dual flow cytometry using combinations of antibodies or markers conveniently conjugated with FITC, PE or PerCP-Cy5.5. Antibody to CD41a was purchased from BD Biosciences (San Jose, CA, USA). Antibody to CD62-P (clone CLBThromb/6) was from Immunotech (Marseille, France). Antibody to factor (F) V/Va was from American Diagnostica Inc (Stamford, CT, USA). Antibody to Fibrinogen (FNG) was from DAKO A/S (Glostrup, Denmark). Anionic phospholipids were detected by annexin V binding from Pharmingen (San Diego, CA, USA). An IgG1 (clone 679.1Mc7) from Immunotech (France) was used as a negative control. Phosphate-buffered saline (PBS) was from Gibco BRL Life Technologies (Barcelona, Spain). Embedding kit JB-4 was from Polyscience (Warrington, PA, USA). Activation of coagulation mechanisms was measured by the kit Enzygnost[®]F1+2micro from Dade Behring (Marburg, Germany). Finally, thrombin generation we measured by Technothrombin TGA (RCL) from Technoclone GmbH (Vienna, Austria).

Aggregation studies

Blood samples were drawn from healthy donors who in the previous 10 days had not taken any drug known to affect platelets or the coagulation system. Blood samples were anticoagulated with CPD at a final concentration of citrate of 19 mM or with LMWH (20 U/ml).

Platelet-rich plasma (PRP) was obtained by centrifugation (120 g, 15 minutes [min]). Aggregation studies with PRP were performed in a four channel aggregometer (APACT 4, Helena Biosciences Europe, Gateshead, UK). Changes in turbidimetric patterns during the interaction of PRP with aggregating agents were registered and results of these changes were expressed as % of maximal aggregation. Aggregation studies were performed using 5-HT (0.5–5 μ M) added to PRP, alone or in combination with ADP (0.5 μ M). Other conventional aggregating agents were tested: ADP (0.5–2 μ M), collagen (Col; 2.5 μ g/ml) and arachidonic acid (AA; 1.4 mM).

The potential antiplatelet effect of citalopram, a selective 5-HT reuptake inhibitor, was also tested in this experimental setting with the same aggregating agents. For this purpose, PRP was incubated with citalopram (2 min, 37°C) at a range of concentrations including some compatible with those reached in clinical conditions (300 nM) as well as higher ones (3 μ M).

Flow cytometry studies

Expression of platelet activation antigens and platelet procoagulant properties were analysed using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) at an excitation wavelength of 488 nm, using dual-color labelling with specific antibodies. Aggregations to 5-HT (5.0 μ M), ADP (0.5 μ M) and 5-HT+ADP, with or without previous incubation with citalopram (300 nM), were stopped at different time points (0, 1 and 5 min) by adding 0.3% paraformaldehyde (final concentration) or by diluting in a Hanks' Balanced Salt solution (HBSS, pH 7.2 containing 3 mM CaCl₂) when analysing annexin V (ANV) binding. Samples were added to polypropylene tubes preloaded with 50 μ l PBS, pH 7.2, incubated with saturating concentrations of FITC- or PE-conjugated antibodies for 15 min, and then diluted with 1 ml PBS for immediate analysis. We used CD62-P, FNG, FV/Va, and binding of ANV to anionic phospholipids as markers for platelet activation and procoagulant properties of platelet membranes.

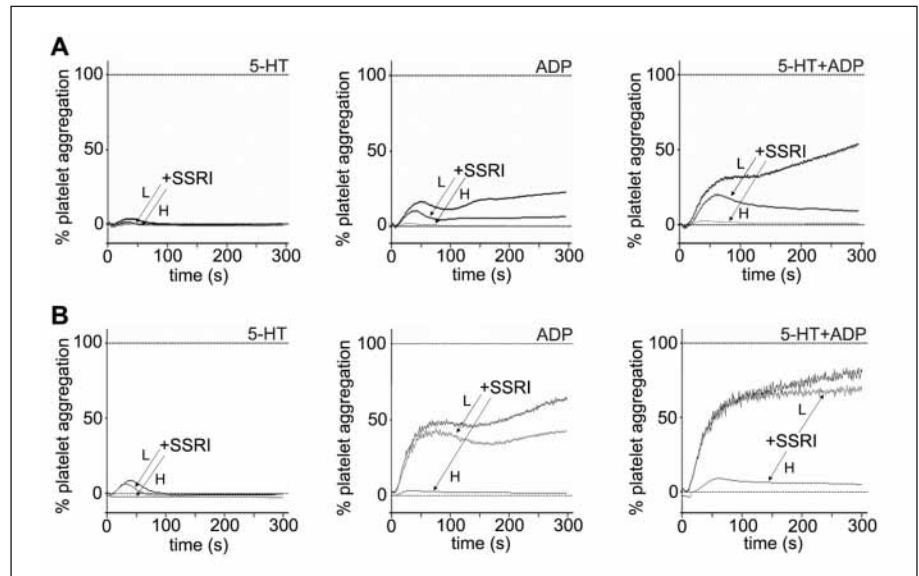
Platelets were differentiated by their characteristic forward versus side scatter and by positivity for the constitutional antigen CD41a. Histograms were composed from fluorescence data obtained in the logarithmic mode from 5,000 events analysed in each sample. A negative control was also performed by using an IgG₁. Data were expressed as the percentage of fluorescence-positive platelets for CD62-P, ANV, FV/FVa and the IgG₁. An analytical marker was set in the corresponding fluorescence channel to define 2% of the resting platelet population with the highest membrane fluorescence at the baseline level. This marker was used as a threshold to determine the proportion of platelets exhibiting immunofluorescence above this level in all subsequent samples.

Thrombin generation assay (TGA)

We evaluated thrombin generation in citrated PRP samples and PRP exposed to 5-HT (5 μ M) with or without previous incubation with Citalopram (300 nM).

Thrombin generation on citrated PRP was assessed with the fluorogenic assay Technothrombin TGA (RCL) from (Technoclone GmbH, Vienna, Austria) following manufacturer's instructions and previous publications (16, 17). This assay is based

Figure 1: Platelet aggregation induced by different agonist using platelet-rich plasma (PRP) obtained from blood anticoagulated with: A) CPD or B) LMWH (20 U/ml). Aggregations were performed in the absence (black line) or in the presence (grey line) of previous incubation with SSRI at clinical dose (L; 300 nM) or at high dose (H; 3 μ M). In both cases, addition of 5-HT to PRP induced a mild reversible aggregation that was more evident when PRP from blood anticoagulated with LMWH was used, suggesting a role of calcium in these aggregations. The combined presence of 5-HT and ADP caused irreversible aggregation of platelets. In all the cases, the presence of SSRI at clinical doses (L) reduced the percentage of aggregation. Platelet aggregation was totally inhibited only after incubation with the higher concentration of SSRI (H) ($n=12$).



on the fluorescence generated by the cleavage of a fluorogenic substrate by thrombin over time upon activation of the coagulation cascade by a reagent (RCL) consisting of low concentration micelles of negatively charged phospholipids containing 71.6 pM of recombinant human tissue factor (TF) and CaCl_2 . Fluorescence generated was measured at 1-min intervals throughout 90 min. The assay provides thrombin concentration as well as other parameters such as the lag time (min) to trigger thrombin generation, peak of maximal thrombin concentration (nM) and time to achieve this peak of thrombin (min). With these parameters, we can obtain a rate of thrombin generation per minute, the velocity index, calculated by the software as follows: [peak thrombin / (peak time-lag time)] expressed in nM/min.

Thromboelastometry studies

In order to evaluate the influence of 5-HT on the whole blood clot formation, we investigated the dynamic thrombelastography of whole blood coagulation, using the ROTEM Thromboelastometry Analyser (Pentapharm GmbH, Munich, Germany) (18). This technique was performed according to the manufacturer's instructions. We used three different tests, the EXTEM, the FIBTEM, and HEPTEM test. In the EXTEM test, TF is used as activator and is sensitive to measure changes on the extrinsic pathway of coagulation, fibrinogen and fibrin polymerisation, and platelet function. In the FIBTEM test platelet function is eliminated with the platelet inhibitor cytochalasin D. While clots obtained in EXTEM are composed of platelets and fibrin, the clot obtained in the FIBTEM assay is primarily a fibrin clot. Finally, the HEPTEM test contains heparinase to evaluate the coagulation system without an effect of heparin if present in the sample.

This technique reports information through different parameters. We have assessed three variables. The clotting time (CT), defined as the time past from the measurement start until the amplitude of the forming clot reaches 2 mm. The clot formation time (CFT) is the time from the start of clot formation until this clot reaches 20 mm of amplitude. Another variable is the clot am-

plitude after 10 min (A10), as a measure of clot firmness. The CT and the CFT indicate the dynamics of clot formation. The clot amplitude gives information about clot strength and stability, which is largely dependent on fibrinogen and platelets.

Attending to our experimental design, some modifications of the standard technique were included. Additionally to the standard determinations in citrated blood, experiments were also performed using blood anticoagulated with LMWH (20 U/ml) from eight different donors. Profiles of continuous whole blood clot formation were recorded for 30 min.

Other modification of the standard technique consisted in the addition of our studied agonists before the addition of the specific reagents of each test. To evaluate the impact of 5-HT in the thromboelastometric properties of blood, we added to the cube a volume of PBS, as a control, or of 5-HT to reach a final concentration of 5 μ M. In those experiments aimed to evaluate the effect of a SSRI, blood samples were previously incubated for 2 min with citalopram (300 nM).

Adhesion studies under flow conditions

Adhesion studies were carried out at 37°C using annular perfusion chambers (19). Enzymatically denuded aorta segments from New Zealand rabbits were mounted inverted on the central plastic rod of the perfusion chamber. Blood samples anticoagulated with LMWH (20 U/ml) were recirculated for 10 min at 37°C using a peristaltic pump (Renal Systems, Minneapolis, MN, USA). Flow conditions were previously adjusted to reach an elevated shear rate equivalent to 1,200 s^{-1} , and a low shear rate equivalent to 300 s^{-1} . Prior to the initiation of perfusions, some blood aliquots were incubated with 5-HT concentrations equivalents to 2 μ M at 37°C for 5 min. The same experiments were also performed with blood samples incubated previously.

After perfusion, segments were rinsed with PBS (0.15 M), fixed with 2.5% glutaraldehyde (in 0.15 M PBS) at 4°C for 24 hours and processed histologically for morphometric evaluation. Fibrin deposition and platelet interaction were evaluated by light microscopy connected to a computer provided with a special

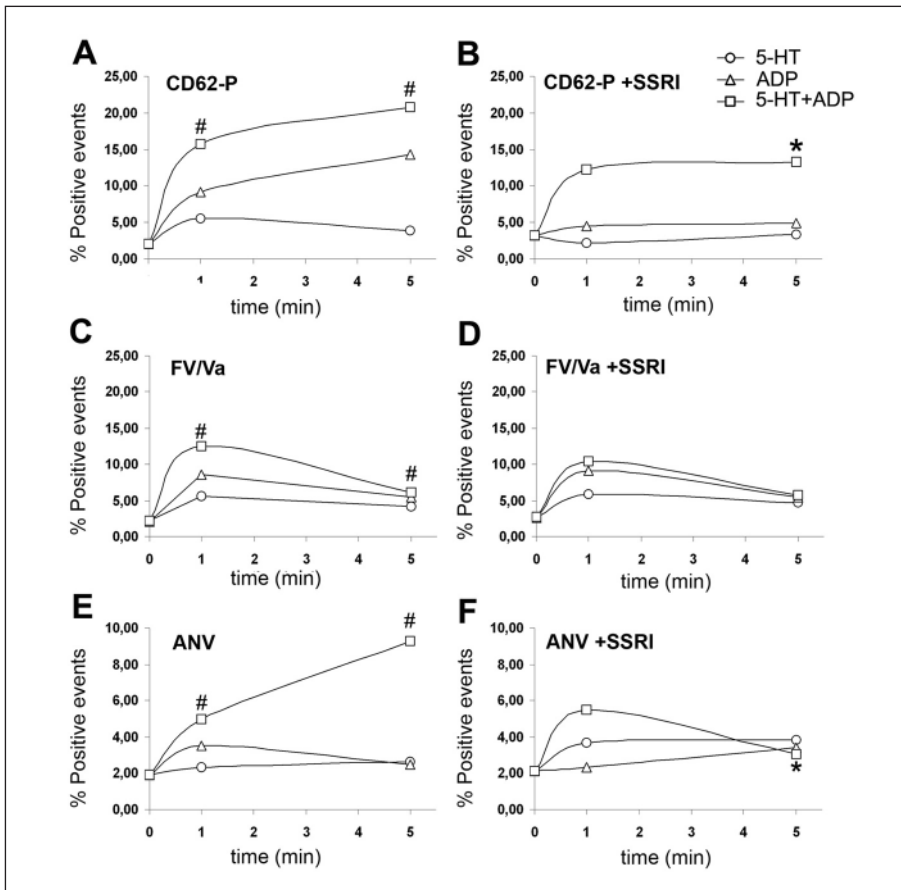


Figure 2: Sequential flow cytometry analysis performed with platelet-rich plasma (PRP) exposed to 5.0 μM of 5-HT (circle, o), 0.5 μM ADP (triangle) or a combination of both agonists 5-HT+ADP (square). Results were expressed as percentage of positive events for CD62-P (A and B), FV/Va (C and D), and annexin V (E and F). PRP was exposed to agonists for 0, 1 and 5 min in the absence of (left column) and after previous incubation with SSRI at clinical dose (300 nM) (right column). 5-HT alone induced a mild activation of platelets when compared to activation with ADP. However, the presence of 5-HT together with ADP increased platelet activation measured as expression of CD62-P and procoagulant properties (FV/Va and annexin V). Previous incubation with a SSRI resulted in a decreased platelet activation (CD62-P) and ANV-binding after 5 min of activation with the combination of ADP+5-HT ($p < 0.05$). (#: $p < 0.05$ vs. time 0 min. *: $p < 0.05$ vs. same point in the absence of SSRI).

Sample	Lag phase	Thrombin peak		Velocity index
	time (min)	thrombin (nM)	time (min)	nM/min
PRP	16.4 ± 2.6	154.4 ± 20.2	21.4 ± 1.4	30.9 ± 4.0
+5-HT	14.4 ± 3.0	201.0 ± 18.0 *	19.7 ± 6.8	37.9 ± 3.4*
+5-HT+ADP	10.5 ± 1.4*†	283.0 ± 54.8*†	15.7 ± 3.0*	54.4 ± 5.4*†
Previous incubation with SSRI				
+5-HT	20.7 ± 13.4	180.7 ± 46.8	26.0 ± 1.4	34.1 ± 12.6
+5-HT+ADP	13.2 ± 2.0	301.7 ± 26.8	18.7 ± 7.4	54.8 ± 14.8

Data expressed as MEAN ± SD (n=4). * $p < 0.01$ vs PRP and † $p < 0.05$ vs 5-HT.

Table 1: Thrombin generation in platelet-rich plasma (PRP) samples exposed to 5-HT and ADP.

software that automatically classifies and quantifies platelet and fibrin coverage (20). Platelet interaction was globally expressed as percentage of surface covered by platelets (% P) and as the mean area of platelet interaction (Area P; μm^2). Furthermore, platelet interaction was classified as contact (C), adhesion (A) or thrombi (T). The presence of fibrin was also morphometrically quantified and expressed as percentage of fibrin (% F) (20).

Activation of coagulation mechanisms

Activation of coagulation during perfusion was monitored through measurements of F1+2 levels in plasma samples. Aliquots of anticoagulated blood were systematically collected before and after perfusion experiments. Aliquots were immediately

mixed with 129 mM sodium citrate to prevent any further activation of the coagulation system. Plasma was separated by centrifugation (14,000 g for 5 min) and frozen at -70°C . Levels of F1+2 were determined in plasma samples using a commercially available enzyme immunoassay (Enzygnost F1+2, Behring, Germany) (21) and expressed in nM.

Statistics

Data are expressed as mean ± standard deviation (SD). The differences among the various conditions were tested by a general linear model for repeated measurements. One-way ANOVA test for independent experiments was applied when multiple comparisons were required, and student's t-test for paired data was

used for comparisons between two different conditions. The level of statistical significance was established at $p < 0.05$.

Results

Aggregation studies

Aggregation studies performed in citrated blood confirmed that 5-HT is a weak agonist for platelets in comparison with standard activating agents. Only the highest concentration of 5-HT tested ($5 \mu\text{M}$) caused minimal and reversible platelet aggregation that was more evident when PRP from blood anticoagulated with LMWH was used ($p < 0.05$) (Fig. 1). Despite this modest effect on aggregation, 5-HT potentiated the aggregation induced by low concentrations of ADP ($0.5 \mu\text{M}$) in PRP samples [$14.6 \pm 8.0\%$ vs. $36.4 \pm 23.1\%$; $p < 0.01$ in samples anticoagulated with CPD ($n = 12$) and $38.0 \pm 12.5\%$ vs. $64.0 \pm 13.8\%$; $p < 0.01$ in samples anticoagulated with LMWH].

Previous incubation of PRP (2 min, 37°C) with citalopram at a concentration equivalent to 300 nM caused statistical reduction in the percentage of maximal aggregation induced by ADP (2 or $0.5 \mu\text{M}$) and ADP+5-HT ($p < 0.05$) (see Fig. 1). Although no statistical inhibition was measured against other aggregating agonists, a tendency to a decrease in the maximal aggregation was observed. Platelet aggregation was totally inhibited with citalopram at concentration 10-fold higher than the clinical dose.

Flow cytometry studies

Flow cytometry studies showed that 5-HT induced a mild activation of platelets when compared to ADP, although combination of both agents resulted in a more potent effect. After 1 min of platelet activation with 5-HT, ADP or 5-HT+ADP, exposure of CD62-P increased in all the cases, but level of statistical significance was only reached when both agonists were present ($p < 0.05$). Activation with ADP or ADP+5-HT increased progressively CD62-P expression up to 5 min, whereas the maximum peak for 5-HT occurred after 1 min, showing a tendency to decrease after 5 min of activation (Fig. 2). ADP and 5-HT+ADP induced FV/FVa release and maximal positive events were obtained at 1 min being significantly higher than 5-HT in both cases, these levels decreased after 5 min of activation in all the cases. Activation with 5-HT showed a mild, but statistical significant increase, in ANV-binding. It was more evident after the incubation with both agonists ADP+5-HT, and was maintained along the 5 min of experiment.

Pre-incubation of samples with citalopram resulted in a decreased platelet activation. Significant reductions up to 40% were observed in the expression of CD62-P and ANV-binding after 5 min of activation with the combination of ADP+5-HT ($p < 0.05$). However, FV/FVa release from alpha granules was barely affected by the previous incubation with citalopram (Fig. 2).

Thrombin generation assay

Thrombin generation in aliquots of control citrated PRP resulted in a maximum thrombin peak of $154.4 \pm 20.2 \text{ nM}$ after $21.4 \pm 1.4 \text{ min}$. Presence of 5-HT increased the velocity index and thrombin generation peak up to $201.0 \pm 18.0 \text{ nM}$ ($p < 0.01$ vs. control) after $19.7 \pm 6.8 \text{ min}$. Previous incubation with a SSRI decreased

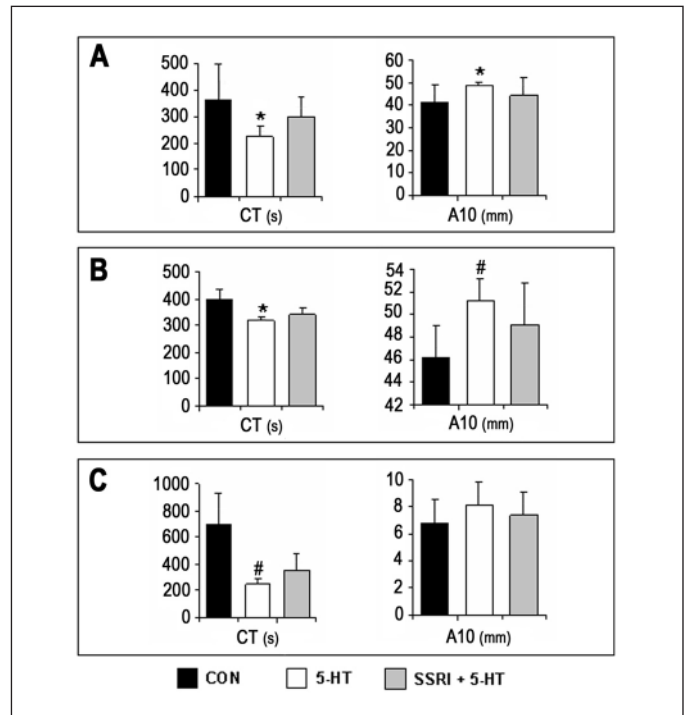


Figure 3: Thromboelastometric analysis of whole blood anti-coagulated with LMWH (20U/ml) exposed to 5-HT without and with previous incubation with a selective serotonin re-uptake inhibitor (SSRI). Each panel corresponds to a different test applied, EXTEM (A), HEPTEM (B) and FIBTEM (C). The parameters represented in each test are the clotting time (CT), expressed in seconds; and the clot amplitude after 10 min (A10) expressed in mm. Bars represent control samples (black), whole blood exposed to $5 \mu\text{M}$ 5-HT (white) and whole blood incubated for 2 min with the SSRI citalopram (300 nM) and following exposed to $5 \mu\text{M}$ 5-HT (grey). Results are expressed as mean \pm SD ($n = 4$); (*) $p < 0.05$ and (#) $p < 0.01$ vs. control samples.

this peak of thrombin generation due to 5-HT. However, the maximal levels of thrombin generation were detected when PRP samples were activated with a mixture of 5-HT+ADP. This increase was statistically significant ($p < 0.01$) with respect to control and to 5HT samples. The previous incubation with the SSRI did not affect thrombin generated in these experimental settings (see Table 1).

Thromboelastometry studies

Studies of thromboelastometry performed in standard conditions using citrated blood did not show differences due to the presence of serotonin or SSRI in any of the test assessed. However, when blood samples were anticoagulated with LMWH differences were evident. 5-HT induced a statistical significant reduction ($p < 0.01$) on the dynamics of clot formation parameters (CT and CFT) and a relevant increase in the clot firmness as revealed the A10 values (see Fig. 3). These effects were constant in all the test used (EXTEM, HEPTEM, and FIBTEM) which indicate a direct effect of 5-HT in fibrin formation, but also in platelet function.

Previous incubation of the blood samples with a SSRI induced a moderate neutralising effect of 5-HT without reaching

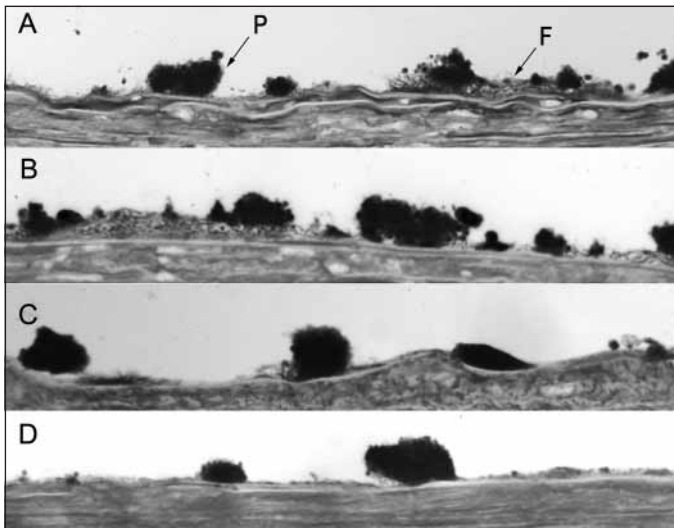


Figure 4: Light micrographs illustrating microscopic fields observed in experiments performed with circulating blood anticoagulated with LMWH, at an elevated shear rate of 1,200 s⁻¹. A) Representative platelet interaction (P) and fibrin formation (F) profiles obtained in control experiments. B) Incubation of whole blood with 5-HT (2 μM), previously to perfusion experiments resulted in a significantly increased formation of large aggregates, while the impact on fibrin formation was more evident at lowest shear rates when compared with control experiments. C) Incubation of blood samples first with a clinical dose of SSRI (300 nM) and second with 5-HT (2 μM) showed a prevention of the enhancing effects of 5-HT, with a reduction in platelet aggregates and a decrease on fibrin deposits on the subendothelium. D) Perfusion experiments with whole blood exposed only to SSRI presented a decrease of covered surface by platelets and also less fibrin deposition indicating the implication of serotonergic mechanisms in these experiments. (Magnification x 400. n≥6).

statistical significance. CT and CFT were slightly increased versus 5-HT, whereas A10 was diminished.

Perfusion studies

5-HT (2 μM) enhanced platelet deposition and fibrin formation in perfusion studies (Fig. 4). The effects of 5-HT were dependent on the shear rate (300 s⁻¹ and 1,200 s⁻¹). In conditions of high shear rate (1,200 s⁻¹), we observed an effect of 5-HT on platelet deposition. Whereas at the lowest rate (300 s⁻¹), the impact of 5-HT was more evident on the fibrin formation.

Perfusion studies at 1,200 s⁻¹, showed an effect of 5-HT increasing the total surface covered by platelets (38.9 ± 6.4 % vs. 26.9 ± 10.6% in control experiments, p<0.05) (Fig. 4A, B). A more detailed analysis of platelet interaction, revealed a significant increase (p<0.05) in the mean area of the large aggregates, with 183.1 ± 31.8 μm² vs. 143.8 ± 28.3 μm² in control experi-

ments. At lower shear rates equivalent to 300 s⁻¹, the presence of 5-HT in the perfusates caused a statistical augment of the fibrin formation (p=0.01), with a percentage of 53.6 ± 5.4% of surface covered by fibrin versus 44.4 ± 6.3% in control samples.

The previous incubation with the SSRI citalopram also neutralised the priming effects of 5-HT on both, platelet interactions and fibrin formation. Citalopram effectively decreased the platelet coverage caused by 5-HT in experiments at high shear rates (30.1 ± 4.9% vs. 38.9 ± 6.4% after 5-HT; p<0.05) (see Fig. 4C). Whereas at the lower shear rate (300 s⁻¹), citalopram significantly diminished the effect of 5-HT on the fibrin formation (21.9 ± 14.0% vs. 53.6 ± 5.2%, p<0.01) with values even below those observed in control experiments (p<0.01). Moreover, the incubation of control samples with the SSRI citalopram at 300 nM, significantly reduced the surface covered by large aggregates, at the higher shear rate tested from 21.6 ± 9.3% to 11.2 ± 6.4% (p<0.05) (Fig. 4D). However, at the lower shear rate, the presence of citalopram in control samples decreased statistically the percentage of fibrin covered surface (20.1 ± 18.1% vs. 44.4 ± 5.9%, p<0.01).

Generation of prothrombin fragment F1+2

Prothrombin becomes activated during perfusion experiments. Table 2 summarises increments in F1+2 levels over baseline values for each experimental condition. Increments in F1+2 levels during perfusion experiments showed a good correspondence with the previous morphometric data. Presence of 5-HT in the perfusates resulted in a consistent enhancement of F1+2 generation throughout the perfusion with differences reaching levels of statistical significance (p<0.05) on studies at 1,200 s⁻¹.

Citalopram effectively inhibited the activation of prothrombin into thrombin caused by 5-HT and significantly reduced thrombin generation below the levels observed in control experiments (see Table 2).

Discussion

Our study has explored the involvement of serotonergic mechanisms in haemostasis by evaluating the effect of 5-HT using different experimental approaches. Data obtained in our experimental model indicate that serotonin (5-HT) primes platelet activation and potentiates procoagulant responses of platelets leading to an enhancement of thrombin generation and accelerated thrombus formation in blood maintained under steady conditions. Even more, 5-HT significantly promoted thrombogenesis in studies with whole blood subjected to flow conditions. All these thrombogenic responses to 5-HT were downregulated through a blockade of the serotonin transporter.

In recent years, clinical evidence has suggested a relationship between involvement of serotonergic mechanisms in depression

Shear rate	Control	5-HT	5-HT+SSRI	SSRI
300 s ⁻¹	0.78 ± 0.23	0.82 ± 0.25	0.51 ± 0.11*‡	0.43 ± 0.25*‡
1200 s ⁻¹	0.60 ± 0.28	1.17 ± 0.65 †	1.03 ± 0.28	0.52 ± 0.37 † §

Results expressed as MEAN ± SD (n=8). * p<0.01 and † p<0.05 vs control perfusates; ‡ p<0.01 vs 5-HT perfusates; § p<0.01 vs 5HT+SSRI perfusates.

Table 2: Generation of pro-thrombin fragment F1+2 on perfusates (nM).

and enhanced risk of cardiovascular events (9, 10, 12, 22). Serotonin (5-HT) has been classically considered a weak platelet agonist. Results of our present studies on platelet aggregation in citrated PRP confirm our previous findings on the synergistic response of 5-HT combined with low concentrations of ADP (8). Moreover, our present data confirm an enhanced expression of CD62-P and an augmented exposure of anionic phospholipids on the platelet membrane. The latter findings share similarities with recent observations indicating the involvement of 5-HT in the development of a subpopulation of platelets, with increased procoagulant activity (23).

It is well known that the aggregatory response to 'weak' agonists depends on calcium ion concentration. Potentiation of 5HT-induced platelet aggregation was more evident when LMWH was used as anticoagulant, confirming that the intensity of platelet responses induced by 5-HT may be influenced by the degree of the calcium chelation induced by the anticoagulant in the sample. Previous studies had suggested that normocalcemia favoured TXA₂ production and TXA₂-dependent secretion during aggregation induced by weak agonists such as ADP or adrenaline (24, 25). The TXA₂ generated in the presence of Ca⁺⁺ is an additional candidate priming platelet activation. Notwithstanding, there is evidence in the literature that inhibition of TXA₂ production by aspirin has little impact on thrombin generation (26, 27). It is well established that thrombin generation and fibrin formation are almost completely inhibited in citrated blood, but fully preserved when LMWH is used as an anticoagulant (28). In our experimental approach, we confirmed that 5-HT increased thrombin generation in the presence of tissue factor in recalcified PRP. 5-HT not only increased peak levels, but also the velocity index of thrombin generation. Presence of 5-HT also shortened clotting times in thromboelastographic studies in blood samples anticoagulated with LMWH. These observations support the concept that effects of 5-HT, and perhaps other weak agonists, on haemostasis would be more evident if studies were performed in the absence of calcium chelators.

The observation that the impact of serotonergic mechanisms on haemostasis is more evident in the presence of Ca⁺⁺ was further reinforced by findings in perfusion studies with whole blood anticoagulated with LMWH conducted under flow conditions. Our results in perfusion studies have demonstrated for the first time, that 5-HT induces a thrombogenic state in circulating human blood. More specifically, 5-HT significantly increases fibrin formation at low shear rate and enhances platelet mediated thrombogenicity on the subendothelium at high shear rates. These results were further confirmed by a significant elevation of F1+2 levels in the perfusates, indicating elevated prothrombin activation in the presence of 5-HT. These observations are in accordance with current knowledge indicating that whereas platelet mediated events (platelet deposition) prevail at high shears, coagulation events (fibrin formation) are more evident at lower shear forces (29).

Similarly to neurons, platelets possess 5-HT_{2A} receptors and serotonin transporters (SERT). It is mainly for these similarities that platelets have been frequently used as a model for the serotonergic neurons (30). Signaling responses triggered through the 5-HT_{2A} receptor have been associated with apparently weak platelet aggregating responses induced by 5-HT (6). Our present

data on 5-HT seem to differ with the idea of 5-HT as a weak agonist. In fact, the differentiation between weak and strong agonists was already challenged by Jin and Kunapuli (7). These authors suggested that physiological responses of platelets may require simultaneous activation through weak and strong receptors, resulting in converging signal transduction pathways that would be responsible for the full functional platelet response. Adrenergic receptors are also considered in the weak side, causing mild platelet activating responses. However, the mild activation caused by minuscule concentrations of epinephrine has demonstrated to reverse the inhibitory action of aspirin on platelet function both *in vitro* (31) and *in vivo* (32). It is worth considering that the enhanced cardiovascular risk referred in patients with mood disorders occurs in a situation with derangement of serotonergic and adrenergic systems (33, 34). Therefore, the difference between weak and strong platelet activating mechanisms should be reconsidered. In this context, our present data suggest that 5-HT could act in combination with other agonists, either present in the circulating blood or released from platelets, to produce more complex responses including the expression of procoagulant proteins.

It is not surprising that inhibitory strategies aimed to the specific blockade of 5-HT_{2A} have proven helpful in the reduction of thrombotic events not only in experimental animal models (35, 36), but also in the clinical setting (37, 38). Moreover, the use of 5-HT_{2A} receptor antagonists is still limited to a synergistic action with SSRIs in major depression treatment (39, 40). In contrast with the pharmacological antagonists of the 5-HT_{2A} receptors, inhibitors of the 5-HT transporter (SERT) are the most frequently prescribed drugs, generally perceived as well tolerated and with a low risk of severe side effects. Citalopram used in our studies is a SSRI with highly selective inhibitory action on SERT (41). SERT is thought to be primarily responsible for the ces-

What is known about this topic?

- Serotonin (5-HT) is a weak agonist.
- Under some conditions 5-HT may induce the development of a subpopulation of platelets with enhanced procoagulant properties.
- The previous concepts have not been further translated into experimental or clinical settings.

What does this paper add?

- Serotonin is not a weak agonist as it has been classically considered.
- 5-HT increases thrombus propagation under flow conditions.
- Our work proves that 5-HT enhances thrombin generation and modifies thromboelastometric properties.
- Inhibition of 5-HT re-uptake, without interfering with the 5-HT_{2A} receptor, reduces the thrombogenic properties induced by 5-HT.
- Selective serotonin re-uptake inhibitors (SSRI) have an effect downregulating the excessive thrombogenicity induced by 5-HT.

sation of action of 5-HT in the central nervous system. In our experimental setting, inhibition of serotonin re-uptake, at concentrations compatible with those achieved in humans, caused effects that would be initially expected from specific antagonists of 5-HT_{2A}. It is interesting to note that presence of a SSRI in PRP reduced rates of platelet aggregation and inhibited the potentiation caused by the combined exposure to 5-HT and ADP. This inhibitory effect was further confirmed by a reduced expression of CD62-P, FV and anionic phospholipids in flow cytometry studies. Moreover, citalopram rapidly and effectively counteracted effects of 5-HT on platelet activation and thrombus formation under flow conditions.

Our data may help to understand the mechanisms through which SSRI interfere with normal haemostasis. Several studies support the rationale for the existence of a link between mood disorders and an enhanced cardiovascular risk (9, 42, 43) considering depression as an independent factor of cardiovascular risk. Recently, some recommendations have been published suggesting the need for screening and treatment of depression in cardiovascular diseases (44). Measurement of 5-HT as an indicator for mood disorders and for the effect of medication (SSRI) have not been reproducible (14, 45, 46). The effect of antidepressant drugs on cardiac risk in some epidemiological studies is also controversial (47). Interestingly, both randomised (48, 49) and non-randomised (50) studies revealed that depressed patients treated with SSRI had reduced cardiovascular risk compared with patients not receiving antidepressant therapy. Furthermore, evidence from the literature has also suggested an increased bleeding risk associated to the use of SSRI (51–53). Our findings provide additional indirect evidence that SSRIs may exert certain antithrombotic activity as previously suggested by Atar et al. (43, 54).

The ability of SSRI to modulate the prothrombotic action of 5-HT observed in our studies can be explained at several levels. First, blockade of SERT could directly prevent phosphorylation of SERT and its membrane exposure (55, 56). Another possible explanation is that blockade of SERT could prevent the amplification of platelet response elicited by released granule contents (57). It has also been documented that associations of the

SNARE protein Syntaxin (Syn1A) with SERT dictates the ability of the transporter to contribute to membrane excitability at neuronal level (58). Moreover, SNARE proteins are known to play a critical role in the regulation of platelet exocytosis (59). Prolonged exposure of patients to SSRI is likely to interfere with the transport of 5-HT into platelet granules thus preventing the development of highly procoagulant platelets. The reduction of cytoplasmic 5-HT concentration is crucial for GTPases activation that trigger platelet alpha granules release (60). Interestingly, a recent publication has suggested an association between SERT and GPIIb-IIIa confirming that GPIIb-IIIa interacts directly with C terminus of SERT (61). This biochemical association SERT / GPIIb-IIIa may also explain the mechanism through which SSRIs could interfere with platelet aggregation mechanisms. Finally, it is expected that chronic inhibition of SERT by SSRIs could induce adaptative response secondary to reuptake inhibition with a downregulation of the number and/or affinity of the serotonergic receptors to 5-HT (40, 50).

Overall, our studies indicate that the contribution of serotonergic mechanisms to the modulation of haemostasis may have been underestimated. Under our experimental conditions, 5-HT potentiated procoagulant responses of platelets and enhanced the thrombogenesis on damaged vascular surfaces. Inhibition of 5-HT reuptake by the SSRI citalopram confirms the implication of serotonergic mechanisms in platelet activation and demonstrated a remarkable antithrombotic action of this drug in our experimental studies. Our results further support that modulators of 5-HT mediated responses may offer a new potential target for the development of antithrombotic strategies. While further investigation is required to discriminate the implication of SERT, serotonin receptors and the role of SSRIs in the haemostatic balance, our present studies on serotonergic mechanisms reinforce the existence of an intriguing connection between mood disorders and cardiovascular risk.

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