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Title: A selective serotonin reuptake inhibitor, citalopram, inhibits collagen-induced platelet aggregation and activation

Authors: Yu-Lun Tseng[a*], Meng-Ling Chiang[b], Tur-Fu Huang[c], Kuan-Pin Su[a,d], Hsien-Yuan Lane[a,e], Yu-Chen Lai[a]

[a]Department of Psychiatry, China Medical University Hospital, Taichung, Taiwan

[b]Department of General Dentistry, Chang Gung Memorial Hospital, Taipei, Taiwan

[c]Department of Pharmacology, Collage of Medicine, National Taiwan University, Taipei, Taiwan

[d]Graduate Institute of Neural and Cognitive Sciences, China Medical University, Taichung, Taiwan

[e]Graduate Institute of Clinical Medical Science, China Medical University, Taichung, Taiwan

***Correspondence:** Yu-Lun Tseng, Department of Psychiatry, China Medical University Hospital, 2 Yuh-Der Road, Taichung, 40447, Taiwan

FAX: 886-4-22052121

TEL: 886-4-22361230

E-mail: triflamp@gmail.com

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Abstract

Clinical depression is a significant risk factor for cardiovascular diseases and confers an increased risk of mortality. Increased platelet reactivity may predispose depressed patients to cardiovascular diseases. The antidepressants selective serotonin reuptake inhibitors (SSRIs) have been found to have cardioprotective effects probably via the attenuation of platelet activation independently in addition to treatment of depression itself. However, the characters of the inhibitory effect of SSRIs on platelets remain largely unknown. Here we show that an SSRI, citalopram, specifically inhibited collagen-induced platelet aggregation. Citalopram, however, revealed only little inhibitory effect on platelet aggregation induced by thrombin, U46619, and ionomycin, and failed to inhibit reversible platelet aggregation induced by adenosine diphosphate with fibrinogen. Collagen-induced of $\alpha_{IIb}\beta_3$ integrin activation in platelets under a static condition was not influenced by citalopram. Citalopram inhibited convulxin-induced platelet aggregation and $\alpha_{IIb}\beta_3$ integrin activation. In the experiments with fibrinogen-induced aggregation in elastase-treated platelets, citalopram inhibited only collagen-induced $\alpha_{IIb}\beta_3$ activation but not the binding activities between activated $\alpha_{IIb}\beta_3$ integrin and fibrinogen. Moreover, citalopram inhibited α-granule and dense granule secretion from platelets in response to collagen, as determined by a reduced expression of P-selectin and adenosine triphosphate release, respectively. In addition, collagen-induced thromboxane A_2 release in platelets was attenuated by citalopram pretreatment. These findings might specify the mechanisms of inhibitory effects of citalopram on collagen mediated platelet activation and aggregation, and further support the cardioprotective effect of SSRIs.

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; c7E3, chimeric 7E3 antibody; GP, glycoprotein; PGE_1, prostaglandin E_1; PRP, platelet-rich plasma; SSRI, selective serotonin reuptake inhibitor; TxA_2, thromboxane A_2; TxB_2, thromboxane B_2.

Key words: citalopram, platelets, collagen, aggregation, granules, thromboxane A_2

Introduction

Numerous studies have provided substantial evidence that depression is associated both with the development and prognosis of cardiovascular disease [1, 2]. Among multiple mechanisms involving in the relationship between depression and cardiovascular diseases, platelet hyperactivity is reported to be a potential biologic abnormality associated with depression [3-5]. The importance of platelet activation and its inhibition is clearly supported by the clinical benefits of using the antiplatelet agents for patients of cardiovascular disease [6]. Interestingly, depression treatments could improve cardiovascular prognosis in patients with coronary artery disease [7]. The selective serotonin reuptake inhibitors (SSRIs) are well established antidepressants with safety profile for patients with heart disease [8, 9]. Moreover, treatment with SSRIs could directly exert inhibitory effects on platelet reactivity [10].

Depression is characterized by serotonin dysfunction in the brain, which could be modulated by recycling through a reuptake and repackaging mechanism [11]. The concept of blockade of the presynaptic reuptake of serotoninin in the brain has led to the development of SSRIs [12], which are now the most widely used antidepressant to treat this condition. In human body, more than 99% of serotonin is stored in platelets [13]. Platelets share biochemistry similarity with central serotonergic neurons in the uptake, storage, and metabolism of serotonin, and have been used as a powerful surrogate to study brain serotonergic synaptosomes [14]. Consistent to the findings from brains, SSRIs also block the reuptake of serotonin into platelet dense granule [15]. It has been reported that a prolonged intake of SSRIs in high doses leads to a significant depletion of intra-platelet serotonin and, subsequently, reduces platelet activation [9, 16, 17].

Platelets respond at the site of vascular injury, where successive phases are involved including an adhesion of glycoprotein Ib (GPIb) and glycoprotein VI (GPVI) to von Willebrand factor and collagen, respectively. The response then triggers the release of granules and thromboxane A_2 (TxA_2) and the activation of integrin $\alpha_{IIb}\beta_3$, which binds to fibrinogen and allows platelet aggregation [18]. Meanwhile, platelet serotonin is released from dense granule during activation. Notably, serotonin is merely a weak platelet agonist that needs adenosine diphosphate (ADP) or epinephrine to enhance its effect. The platelet-release serotonin has an established role in aiding in hemostasis by a regulation of vasoconstriction or dilation rather than by the direct influence of platelet aggregation [13]. Given that serotonin is a weak agonist for the platelet aggregation, it is too simple to attribute the inhibitory effect of SSRIs on platelet aggregation to the depletion of intra-platelet serotonin after a prolong treatment of SSRIs. Moreover, the role of SSRIs involved in the multiple phases of the response of platelets to agonists remains largely unknown. In this study, we found that citalopram, one of the widely used SSRIs, specifically inhibits collagen-induced platelet aggregation in the experiments with isolated platelet preparation. We further characterized the inhibitory effects of citalopram on the granule secretion and TxA_2 released by platelets in response to collagen challenges.

Methods and materials

Antibodies and reagents

Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human $\alpha_{IIb}\beta_3$ integrin antibody PAC-1 and FITC-conjugated mouse monoclonal anti-human P-selectin antibody AK-4 were purchased from Becton Dickinson (San Diego, CA, USA). Anti-human integrin $\alpha_{IIb}\beta_3$ chimeric 7E3 antibody (c7E, ReoPro) was kindly supplied by Eli Lilly, Taiwan. All antibodies were used at optimal concentrations, as determined by titration.

Citalopram, human thrombin, adenosine diphosphate (ADP), ionomycin, elastase (type IV), bovine serum albumin (BSA), prostaglandin E_1 (PGE_1) and collagen (type I, bovine Achilles tendon) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Convulxin was purchased from LOXO (Dossenheim, Germany). U46619 was from Cayman Chemical (Ann Arbor, MI, USA). Heparin was from LEO Pharma (Ballerup, Denmark). BCECF-AM was from Molecular Probes (Leiden, The Netherlands). Other chemicals were purchased from Sigma-Aldrich or Wako (Osaka, Japan).

Preparation of platelet suspension and elastase-treated platelet suspension

Human platelets were purified as previously described [19]. This study was approved by the Committee of Ethics of China Medical University Hospital. In brief, blood from healthy donors was collected by venipuncture into acid-citrate-dextrose (9:1) and centrifuged at 200 × g for 20 min at 25°C to prepare platelet-rich plasma (PRP). PRP then was firstly washed with modified Tyrode's solution (NaH_2PO_4, 0.4 mM; NaCl, 136.9 mM, KCl 2.7 mM; $NaHCO_3$, 11.9 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM; 0.35% BSA and 0.1% glucose) containing heparin (7 U/ml) and PGE_1 (6 nM), and

centrifuged at 500 × g for 15 min at 25°C. After descanting the supernatant, pellet was then washed twice with modified Tyrode's solution containing heparin and PGE_1. Finally, washed platelet were resuspended to a final concentration of 3×10^8 platelets/ml in Tyrode's solution containing 0.35% BSA and incubated at 37°C for use.

Elastase-treated platelets were prepared as previously described by Komecki [20]. Briefly, human platelet suspensions were incubated with elastase (10 U/ml) for 1 h at 37°C. Platelets were then washed three times by centrifugation and resuspended in modified Tyrode's solution.

Platelet aggregation assay

Platelet aggregation was measured with an aggregometer (Payton Scientific, Buffalo, NY, USA) as previously described [21]. Briefly, platelet suspension at concentration of 3.5×10^7 per millimeter was applied to the aggregometer and stirring was initiated at 900 rpm for 1 min at 37°C with a small magnetic bar. Then various concentrations of indicated citalopram were added and incubated for 3 min followed by adding proaggregatory substance thrombin (1 U/ml), U46619 (10 μM), collagen (10 μg/ml), ADP (20 μM), ionomycin (10 μM) or convulxin (200 ng/ml). We used PowerLab 8/SP (ADInsturments, Sydney, Australia) to analyze the extent of platelet aggregation that was continuously monitored for 8 min by turbidimetry and expressed as increase of light transmission.

Flow cytometry

The expression of activated integrin $\alpha_{IIb}\beta_3$ and P-selectin was performed by using cytofluorimetric analysis. All reactions were performed with 100 μl total volume

containing 1 $\times 10^6$ isolated platelets. In some experimental conditions, c7E3 (10 μg/ml) was added to inhibit platelet aggregation. After pretreatment with indicated concentrations of vehicle solution, citalopram, c7E3 or PGE_1 for 3 min at 37°C, isolated platelets were stimulated by the agonist collagen (10 μg/ml) or convulxin (200 ng/ml) and then incubated for 10 min. After extensive washes, platelets were labeled with monoclonal antibodies raised against P-selectin (AK-4) or activated integrin $\alpha_{IIb}\beta_3$ (PAC-1) at room temperature for 30 min. Cells then washed, resuspended in phosphate buffer saline, and analyzed immediately by FACSCanto (Becton Dickinson, USA).

Platelet adhesion assay

Ninety-six flat-bottom well microliter plates were coated with fibrillar collagen or fibrinogen (50 μg/ml) at 4°C overnight. Wells were then blocked with 1% BSA in PBS for 1 h at 37°C. Platelets were labeled with fluorescent dye BCECF-AM for 30 min. After a brief wash, platelets were allowed to seed with various concentration of citalopram for 1 h at 37°C. At the end of the incubation, non-adherent cells were removed by a brief wash with PBS. Attached platelets were read by a Cytofluor microplate reader with fluorescence excitation and emission wavelength at 485 nm and 530 nm, respectively. Adhesion of platelets was quantified as the percentage of fluorescence intensity of control platelets.

Measurement of ATP release

A luciferin-lucifease detection system, adenosine triphosphate (ATP) Bioluminescent Assay Kit (Sigma-Aldrich), was used to quantify ATP release of platelets. The platelets were pretreated with various concentrations of citalopram or with vehicle

solution for 3 min at 37°C, and stimulated with collagen (10 μg/ml) under a stirring condition. In some experimental conditions, c7E3 (10 μg/ml) was added to inhibit platelet aggregation. At indicated time points the samples were centrifugated at 14000 cpm for 30 sec at room temperature. After adding an equal volume of luciferin-luciferase solution to the supernatant, chemoluminescence was determined immediately using a luminometer (Modulus ^{TM}; Turner BioSystems, Sunnyvale, CA, USA).

Measurement of TxA_2 formation

The platelets were pretreated with various concentrations of citalopram or with vehicle solution for 3 min at 37°C, and stimulated with collagen (10 μg/ml) under stirring conditions. In some experimental conditions, c7E3 (10 μg/ml) was added to inhibit platelet aggregation. The reaction was terminated by the addition of indomethacin (50 μM) and EDTA (2 mM) at indicated time points and the samples were centrifugated at 3000 × g for 2 min at 4°C. The content of thromboxane B_2 (TxB_2), the stable metabolite of TxA_2, in the supernatant was determined using a TxB_2 enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Statistical analysis

All values are presented as mean ± SEM. Analysis of three or more groups was performed by using one-way ANOVA and Newman-Keuls multiple comparison test. Two groups were compared by Student's t test. P values less than 0.05 ($P<0.05$) were considered significant.

Results

Citalopram inhibits collagen-induced platelet aggregation

To examine the direct antiplatelet effect of citalopram, we performed *in vitro* platelet aggregation studies. Platelet suspension was incubated with citalopram followed by adding agonists to induce platelet aggregation. Citalopram fully inhibited collagen-induced platelet aggregation, whereas platelet aggregation in response to thrombin, the TxA_2 analog U46619, and calcium ionophore ionomycin was marginally inhibited by citalopram. In ADP-induced platelet aggregation assay, the platelet suspension was pretreated with fibrinogen because ADP solely failed to elicit aggregation in isolated platelet preparation. The primary phase of ADP-induced platelet aggregation was not inhibited by citalopram (Fig. 1A). In addition, citalopram did not inhibit ristocetin-induced GPIb-dependent platelet agglutination (data not shown). Citalopram inhibited collagen-induced platelet aggregation in a concentration-dependent manner (Fig. 1B). The concentration of citalopram required for half-maximal inhibition (IC_{50}) of collagen-induced platelet aggregation was 23.4 μM (Fig. 1C). Notably, the platelet shape change, which was demonstrated in the aggregometry as initial decrease of light transmission following the activation of collagen, was delayed by pretreatment of citalopram (Fig. 1A and B). By contrast, citalopram failed to influence initial platelet shape change activated by other tested agonists. Together, these findings suggest that citalopram specifically inhibits collagen-induced platelet aggregation and activation.

Citalopram inhibits GPVI agonist-induced platelet aggregation and PAC-1binding

We next examined the effect of citalopram on integrin $\alpha_{IIb}\beta_3$ activation of platelets. Platelet suspension was incubated in static condition with collagen. As depicted in

immunofluorescence staining with anti-$\alpha_{IIb}\beta_3$ integrin monoclonal antibody PAC-1, which recognizes an epitope on the activated integrin $\alpha_{IIb}\beta_3$ of platelets, collagen significantly elicited activation of integrin $\alpha_{IIb}\beta_3$ on platelets (Fig 2A and 2B). In addition, citalopram did not influence binding of PAC-1 on resting platelets (Fig. 2B). Unexpectedly, the collagen-induced increment of PAC-1 binding was not affected by citalopram (Fig. 2A and 2B). We used c7E3 to confirm that the binding of PAC-1 to integrin $\alpha_{IIb}\beta_3$ was specific. Moreover, collagen-induced binding of PAC-1 under this condition could be inhibited by PGE_1 (Fig. 2B), indicating the validity of this experiment. These results imply that citalopram failed to inhibit collagen-induced neoepitope expression of integrin $\alpha_{IIb}\beta_3$ being in this static condition.

GPVI is the major receptor on the platelet membrane in initiation of collagen binding and mediated platelet activation [22]. As shown in Fig. 3A, platelet aggregation induced by convulxin, a GPVI agonist, was inhibited by pretreatment of citalopram. Moreover, convulxin-induced PAC-1 binding was reduced by citalopram (Fig. 3B), indicating that citalopram inhibited GPVI-mediated platelet activity.

Effects of citalopram on the binding of fibrinogen or collagen to platelets

If citalopram cound not inhibt collagen-induced integrin $\alpha_{IIb}\beta_3$ activation, the inhibitory effect of citalporam on platelet aggregation could be due to influence of interaction between fibrinogen and activated integrin $\alpha_{IIb}\beta_3$. To test this possibility, we tested the effect of citalopram on elastase-treated platelets. The activation of integrin $\alpha_{IIb}\beta_3$ on platelets was bypassed by pretreated with elastase. Thus, elastase-treated platelets aggregated spontaneously upon the addition of fibrinogen. Pretreatment of citalopram failed to inhibit fibrinogen-induced aggregation of elastase-treated platelets (Fig. 4A), indicating that citalopram did not directly

influence the binding between fibrinogen and integrin $\alpha_{IIb}\beta_3$. In contrast to ordinary platelet suspension, elastase-treated platelets failed to aggregate in response to adding collagen but robustly aggregate following in addition of fibrinogen. Pretreatment of citalopram inhibited collagen-induced platelet aggregation but still permitted fibrinogen-mediated aggregation (Fig. 4B). Moreover, initial platelet shape change was abolished under this condition. This further suggests an inhibitory effect on collagen-platelet interactions rather than a direct influence on integrin $\alpha_{IIb}\beta_3$-dependent aggregation.

We further investigated the effects of citalopram on the binding of collagen or fibrinogen to platelets by using an adhesion assay. Citalopram did not block the adhesion of platelets to immobilized collagen and fibrinogen (data not shown).

Citalopram inhibits collagen-induced platelet activation

Upon exposure to collagen, platelets undergo a morphologic transformation that includes shape change and loss of platelet granules [18]. Based on the observation from platelet aggregometry that platelet shape change in response to collagen was attenuated and slower, we speculated that citalopram might influence granule secretion stimulated by collagen. Therefore, we determined the degree of α-granule secretion by analysis of P-selectin expression over time by flow cytometry. As depicted in Fig. 5, collagen-induced P-selectin expression was inhibited and decelerated by citalopram in a concentration-dependent manner.

The effect of citalopram on collagen-induced dense granule secretion was monitored according to ATP release in a stirring condition. Citalopram inhibited ATP release in response to collagen. Similarly, c7E3 also inhibited collagen-induced ATP release in the same degree (Fig. 6A). Thus, inhibition of collagen-induced dense

granule might be caused by the inhibitory effect of citalopram on platelet aggregation. In the presence of c7E3, which inhibits platelet aggregation, citalopram decelerated and still inhibited agonist-induced ATP release (Fig. 6B). Together these findings indicate that citalopram is capable of directly inhibiting collagen-induced platelet activation rather than the responsiveness secondary to collagen-induced aggregation.

Citalopram inhibits collagen-induced TxA_2 formation

It is well known that collagen induces platelet aggregation through a pathway that is primarily mediated by the release of TxA_2 [22, 23]. Therefore, we determined the effect of citalopram on the production of TxB_2 in response to collagen. As expected, we found that citalopram significantly inhibited the secretion of TxB_2 under a stirring condition (Fig. 7). Notably, c7E3 also showed the inhibitory effect on TxB_2 release in the stirring condition, which suggested that platelet aggregation itself also contributed to collagen-induced TxB_2 release. In the presence of c7E3, collagen-induced TxB_2 release by platelets was further blocked by citalopram. Citalopram itself showed no effect on the secretion of TxB_2 by platelets. Taken together, these data clearly indicate that citalopram directly inhibits collagen-induced platelet TxA_2 release but only partially inhibits the responsiveness secondary to collagen-induced aggregation.

Discussion

To our knowledge, this is the first study to demonstrate an agonist-dependent role for citalopram in the inhibitory effect on platelet aggregation. Under the condition of isolated platelet preparation, citalopram specifically inhibited collagen-induced aggregation, whereas had little inhibitory effects on aggregation elicited by ADP or thrombin.

The findings of SSRIs' effects on platelet aggregation are inconsistent. In one study, the collagen/epinephrine-induced closure time measured by platelet functional analyzer-100 was prolonged in whole bloods sampled from in patients treated with paroxetine [16]. Another study, however, did not find the difference in closure time was found in patients treated with escitalopram except in high dose [24]. In PRP, platelet aggregation in response of collagen was decreased in patients using SSRIs comparing with the bupropion group that did not have inhibitory effect, but aggregation elicited by ADP was not inhibited [25]. Another study revealed that escitalopram pretreatment in PRP inhibited the platelet aggregation induced by ADP and collagen [24]. Maurer-Spurej et al. showed that SSRIs did not affect platelet aggregation in response to thrombin, but significantly reduced ADP-induced second wave of platelet aggregation [26]. Similarly, Serebruany et al. revealed that pretreatment of blood sample with sertraline resulted in a dose-dependent inhibition of PRP aggregation triggered by ADP, collagen and thrombin [27]. Some of these conflicting findings may be due to inconsistencies in research methodology. Although physiological condition is reserved in whole blood sample and PRP, the complexity under these conditions influences the way to delineate each agonist-dependent pathway. In addition, it is known that SSRIs differ in efficiency in treating depression

[28], implicating that there are different pharmacological properties between these SSRIs. Comparing to other SSRIs, citalopram is a much pure serotonin reuptake inhibitor and has lesser degrees of secondary actions at other receptors or transporters [29]. In our experiments, citalopram failed to inhibit the primary phase of ADP-induced aggregation in isolated platelet preparation, whereas citalopram inhibited the second phase of ADP-induced aggregation in PRP (data not shown), indicating that the inhibitory effect of citalopram on ADP-induced aggregation is not straightforward. On the contrary, under the condition of isolated platelet preparation which excluded plasma factors such as von Willebrand factor, which act synergistically with collagen to stimulate platelets, we provided direct evidence that a pathway related to reuptake of serotonin is essential for collagen-induced platelet aggregation.

Platelet aggregation is mediated by adhesive substrate, notably fibrinogen, bound to the activated $\alpha_{IIb}\beta_3$ integrin on platelets [18]. It was suggested that if SSRIs have antiplatelet effect, they could inhibit the activation of $\alpha_{IIb}\beta_3$. Indeed, activation of $\alpha_{IIb}\beta_3$ integrin was not changed in two clinical studies for escitalopram [24, 30]. In addition, the study of sertraline exhibited only minimal change of fibrinogen receptor binding [9]. Given that platelets aggregate after adding agonist in the aggregation assay, it is difficult to evaluate the activation of $\alpha_{IIb}\beta_3$ integrin by using cytofluometric assay in this condition. Hence, we examined collagen- and convulxin-induced activation of $\alpha_{IIb}\beta_3$ integrin by platelets under a static condition, and found that citalopram inhibited GPVI-mediated expression of $\alpha_{IIb}\beta_3$ integrin. However, citalopram did not influence the activation of $\alpha_{IIb}\beta_3$ integrin in response to collagen. This paradoxical result is speculated that collagen mediates distinct pathways for activation of $\alpha_{IIb}\beta_3$ integrin under different experimental conditions. It is known that platelet interact with collagen through adhesion molecules including GPIb, GPVI,

and $\alpha_2\beta_1$ integrin [22]. GPIb and GPVI can bind to fibrillar collagen even without prior activation, while $\alpha_2\beta_1$ integrin requires at least some level of activation to expose their binding sites for collagen [31]. Therefore, we suggested that under the static condition, collagen binds to a distinct receptor and triggers a signaling pathway that bypass the influence of citalopram. Regarding the influence of citalopram on $\alpha_2\beta_1$ integrin pathway, a snake venom peptide, aggretin, with $\alpha_2\beta_1$ integrin agonistic property [32], would be the research tool to address this issue.

In our experiments, lack of the inhibitory effect of citalopram on fibrinogen-mediated aggregation of elastase-treated platelets, indicating that citalopram did not block the binding between activated $\alpha_{IIb}\beta_3$ integrin and fibrinogen. In the same condition, however, we found citalopram attenuated the initial shape change of platelets elicited by collagen and inhibited fibrinogen-mediated aggregation. These findings implied that citalopram could influence an inside-out signal triggered by collagen and also the subsequent outside-in pathway mediated by binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$. Recently, Carneiro and colleagues reported that acute pretreatment of washed human platelets with citalopram diminishes the spreading of platelets onto fibrinogen [33]. In addition, knockout study indicates a more prominent role of the requirement of ongoing serotonin transport by serotonin transporters in maintaining fibrinogen-mediated platelet aggregation [33]. Binding of collagen to receptors on platelets triggers a signaling to produce TxA_2. Released TxA_2, in turn, binds to its receptor and results in the activation of $\alpha_{IIb}\beta_3$ integrin. Moreover, binding of fibrinogen to activated $\alpha_{IIb}\beta_3$ integrin induces a signal that leads to a regulatory positive feedback involving TxA_2 release, which exaggerates the aggregation induced by collagen [34]. We identified that citalopram inhibits TxA_2 release in an aggregated condition that elicited by collagen. After blocking the aggregation by c7E3, we

further confirmed the inhibitory effect of citalopram on collagen-induced TxA_2 release. Together these findings, we suggested that citalopram would inhibit platelet aggregation induced by collagen itself as well as the subsequent aggregation reinforced by the binding of fibrinogen.

Consistent with the result of most studies that assessing the effects of SSRIs on platelet parameter [9, 16, 17], we identified that platelet α-granule and dense granule secretion were inhibited by citalopram. The limitation of this study is that platelet serotonin levels were not measured. Generally, loading of radiolabeled serotonin requires incubation of platelets with 2 μM concentration of serotonin for 30 min at 37°C [14]. Given that this *in vitro* study incubated sample with citalopram immediately before assessing platelet function, we doubt if citalopram could influence intra-platelet serotonin level during several minutes in isolated platelet preparation under this condition. In other words, we suggested that the citalopram could inhibit the serotonin transporters, which interact with integrin $\alpha_{IIb}\beta_3$ on platelet [33], and regulate platelet aggregation. The other possibility is that serotonin transporter itself triggers signaling pathway by the transport of serotonin, thus regulates the activation of platelets in response to collagen. The involvement of p38 mitogen-activated protein kinase pathway [35], which is a marker of collagen-induced aggregation, is an intriguing focus for study. However, further studies are required to confirm this idea. In addition, micromolar concentrations were required for platelet inhibition in this study, while therapeutically achieved concentration of citalopram are much lower [36]. Therefore, other cellular effects than serotonin transporter antagonism at higher concentration of citalopram is possible. There is evidence that citalopram at micromolar concentrations influences platelet phospholipase A_2 activity and

decreases arachidonic acid liberation through intercalation in membrane phospholipids [37].

In conclusion, citalopram significantly inhibits collagen-induced platelet aggregation and activation in isolated platelet preparation. Although we only investigated citalopram, we think this finding can be inferred to other SSRIs. Clarification of the exact role of SSRIs on platelet functions is needed to understand the potential benefits of these antidepressants for the patients with depression and cardiovascular disease.

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Fig. 1. Inhibitory effects of citalopram on platelet aggregation. (A) Platelet suspension was preincubated with buffer (gray lines) or citalopram (50 μM, black lines) at 37°C for 3 min, and then thrombin (1 U/ml), U46619 (10 μM), collagen (10 μg/ml), ADP (20 μM) plus fibrinogen (200 μg/ml) or ionomycin (10 μM) were added to trigger aggregation. (B) Concentration-dependent inhibitory effect of citalopram on platelet aggregation triggered by collagen (10 μg/ml). These experiments are representative results of at least three similar experiments. (C) Concentration-inhibition curve of citalopram on collagen-induced platelet aggregation. Data are means of three independent experiments.

Fig. 2. Flow cytometric analysis of the effects of citalopram on the activation of integrin $\alpha_{IIb}\beta_3$ by platelets after stimulation with collagen under static condition. (A) Platelet suspension was pretreated with buffer (solid line) or citalopram (50 μM, gray area) before adding collagen (10 μg/ml), then probed with anti-$\alpha_{IIb}\beta_3$ integrin antibody (PAC-1). The expression of integrin $\alpha_{IIb}\beta_3$ by resting platelets is shown as dotted line. (B) Quantitative analysis of the influence of citalopram (50 μM), c7E3 (10 μg/ml) or PGE_1 (10 μM) on the expression of activated integrin $\alpha_{IIb}\beta_3$ by resting (open bars) and collagen-stimulated platelets (filled bars) by means of the mean fluorescence intensity of PAC-1. * $p<0.01$ vs. resting platelets. ** $p<0.01$ vs. control sample of collagen-induced platelets.

Fig. 3. Inhibitory effects of citalopram on GPVI-mediated platelet aggregation and activation of integrin $\alpha_{IIb}\beta_3$. (A) Platelet suspension was pretreated with buffer or citalopram (50 μM) at 37°C for 3 min, and then convulxin (200 ng/ml, arrow) was added to trigger aggregation (B) Platelet suspension was pretreated with buffer (thick

line) or citalopram (50 μM, gray area) before adding convulxin (200 ng/ml), then probed with anti-$\alpha_{IIb}\beta_3$ integrin antibody (PAC-1). The expression of integrin $\alpha_{IIb}\beta_3$ by resting platelets is shown as thin line.

Fig. 4. Effects of citalopram on fibrinogen-mediated aggregation of elastase-treated platelets. (A) The aggregation was initiated by the addition of fibrinogen (200 μg/ml, arrow head). Comparing with control, citalopram (50 μM) did not influence platelet aggregation under this condition. (B) Citalopram (50 μM) inhibited fibrinogen-mediated aggregation in elastase-treated platelets triggered by collagen (10 μg/ml, arrow). The initial decrease in transmittance (black bar) demonstrates platelet shape change occurred after stimulation by collagen, which was attenuated in citalopram-pretreated platelet preparation. These results were confirmed in 3 separate experiments.

Fig. 5. Effects of citalopram on platelet α-granule secretion elicited by collagen. (A) In the presence of c7E3, platelets were pretreated with vehicle (○), 5 μM citalopram (▲), or 50 μM citalopram (●) for 3 min. Platelets were then stimulated with collagen (10 μg/ml, arrow) under stirring condition for 8 min, and subsequently terminated the reactions by adding cold buffer at the indicated times. Samples were then probed with anti-P-selectin antibody and assayed the P-selectin immunoreactivity (mean fluorescence intensity) by flow cytometry. Inset: resting platelets (dotted line) was negative control. Collagen-induced platelets (solid line) expressed high level of P-selection, and citalopram inhibited it (gray area).

Fig. 6. Effects of citalopram on platelet dense granule secretion elicited by collagen. (A) Concentration-inhibitory curve of citalopram on platelet ATP release elicited by collagen (10 μg/ml) under a stirring condition. Alternatively, c7E3 (10 μg/ml, open circle) was added to examine the contribution of aggregation itself in this reaction. (B) In the presence of c7E3, platelets were pretreated with vehicle (○), 5 μM citalopram (▲), or 50 μM citalopram (●) for 3 min. Platelets were then stimulated with collagen (10 μg/ml, arrow) under stirring condition for 8 min, and subsequently assayed for ATP release using a luciferin-luciferase detection system at indicated times. Data are expressed as relative light units (mean ± SEM) of three independent experiments.

Fig. 7. Citalopram inhibited TxB_2 generation by collagen-stimulated platelets. The platelets were incubated with vehicle solution (control) or citalopram (50 μM) for 3 min under a stirring condition with c7E3 (black bars) or not (open bars) prior to stimulation with collagen. The supernatants were then analyzed for TxB_2, the stable metabolite of TxA_2, by EIA. * $p<0.01$ vs. control.