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Calcium mobilization by the plant estrogen ferutinin does not induce blood platelet aggregation

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Abstract:
Platelet activation is closely associated with an increase in intracellular Ca^{2+} concentration. Various compounds including Ca^{2+} ionophores are able to trigger platelet aggregation by increasing intracellular Ca^{2+} concentration in platelets. In the present study, we monitored the effect of the phytoestrogen ferutinin, which acts as a Ca^{2+} ionophore in human blood platelets; its ionophore-like properties include upregulation of $[Ca^{2+}]_{in}$, activation of fibrinogen receptors and increased fibrinogen binding. Using spectrofluorometry and triple-color flow cytometry, we demonstrate that ferutinin increases $[Ca^{2+}]_{in}$ in both isolated platelets and platelets in whole blood from humans. This effect was almost completely blocked by the Ca^{2+} chelator EGTA and was not sensitive to either Gd^{3+} or econazole, which inhibit VOC and SOC channels, respectively. Nor was the effect sensitive to thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+} ATPases. Ferutinin stimulated the expression of the active form of the GPIIb-IIIa complex and whole blood platelet aggregation only weakly and had no statistically significant effect on the binding of fibrinogen. These results demonstrate apparently inconsistent effects of ferutinin, which raises intraplatelet Ca^{2+} concentration but fails to have an effect on spontaneous blood platelet aggregation. This pattern of responses may be caused by the combination of ferutinin's Ca^{2+} ionophoric and estrogenic properties.

Key words:
phytoestrogen, ferutinin, platelets, calcium

Introduction

Ferutinin is a plant-derived ester of sesquiterpenic alcohol with aromatic (*p*-hydroxybenzoic) acid, which is isolated from *Ferula tenuisecta* Korov and displays high estrogenic activity [15]. The estrogenic activity of ferutinin is the result of its ability to bind to estrogen receptors with a high affinity, a characteristic that is underlined by the structural similarities between ferutinin and steroid hormones [4, 16].

Recently, experiments involving planar lipid membranes (PLM) have shown that ferutinin has electrogenic Ca^{2+} ionophore activity [2, 38]. Investigations of the actions of ferutinin on intracellular calcium homeostasis in various cell types (thymocytes, hepatocytes, neurons and Jurkat cells have shown that this agent increases the cytosolic Ca^{2+} level [1, 20, 38]. However, the effect of Ca^{2+} elevation by ferutinin in hepatocytes and neurons was different from that of the more commonly used Ca^{2+} ionophores A23187 and ionomycin. In contrast to classical Ca^{2+} ionophores, which lead to the collapse of ionic gradients across the membranes of various intracellular compartments, ferutinin induced increased concentrations of cytosolic Ca^{2+} and a subsequent accumulation of Ca^{2+} in mitochondria [1]. In Jurkat cells, ferutinin also induced a biphasic elevation in intracellular Ca^{2+} that was completely blocked by nifedipine and mostly blocked by verapamil [20].

The discovery of the stimulatory effects of ferutinin on Ca^{2+} concentrations in various cell types encouraged us to speculate on the possible effects of ferutinin on platelet activation. It is well known that increases in intracellular Ca^{2+} play a pivotal role in various phases of platelet activation, such as shape change, secretion, cytoskeleton contraction and aggregation [32]. A wide spectrum of pharmaceuticals, including steroid hormones, have been shown to positively affect the functional status of blood platelets, thereby increasing the risk of cardiovascular diseases. Some of these agents have been reported to inhibit or potentiate the effects of natural physiological factors without inducing platelet aggregation by themselves [24, 25, 28]; others can directly induce platelet aggregation by increasing intracellular Ca^{2+} concentration [10].

It is known that some Ca^{2+} ionophores, such as ionomycin and A23187, are able to stimulate platelet activation at all stages [10, 14, 21]. However, numerous compounds that have been described as Ca^{2+} ionophores (based on investigations in either artificial or natural systems) are in some cases unable to activate blood platelets [21].

In this paper, we studied the actions of ferutinin on various parameters of platelet signaling and reactivity, with the aim of shedding new light on the association(s) between ferutinin's ability to increase intracellular Ca^{2+} and its effects on the pathways that trigger platelet activation.

Materials and Methods

Plant material

Roots from *Ferula tenuisecta* Eug. Korov (*Apiaceae*) were collected in the valley of Tashkent (Uzbekistan) and identified by Dr. A.M. Nigmatullaev (Laboratory of Medical Plants, S.Yu. Yunusov Institute of Chemistry of Plant Substances, Tashkent, Uzbekistan). A sample specimen has been deposited in the herbarium of the Yunusov Institute.

Reagents

Gadolinium chloride, econazole (1-{2-[(4-chlorophenyl)-methoxy]-2-(2,4-dichlorophenyl)-ethyl}-1*H*-imidazole), ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, tetrasodium salt ($EGTANa_4$) and other chemicals were from Sigma-Aldrich Co. (USA). Fura-2 acetomethoxyl ester (Fura-2 AM) and Fluo-3-acetomethoxyl ester (Fluo3-AM) were from Molecular Probes (The Netherlands). Oregon Green® 488 conjugated-fibrinogen derived from human plasma was from Molecular Probes, Inc., USA. CD61/PerCP (mouse monoclonal antibody against human platelet CD61), PAC-1/FITC, Cell Wash and CellFix (phosphate buffered fixative containing 10% v/v formaldehyde and 1% w/v sodium azide) were from Becton Dickinson (Germany). Ferutinin was prepared by Dr. A. Saidkhodjaev (Institute of Chemistry of Plant Substances, Tashkent, Uzbekistan) as previously described [33].

Tubes for blood collection containing 0.105 M sodium citrate (*Vacutainer tubes*) were from BD Diagnostics (Plymouth, UK). Water used for solution preparation and glassware washing was passed through an Easy Pure UF water purification unit (Thermolyne Barnstead, IA, USA).

Isolation and mass spectrometric identification of ferutinin

Methanol solutions of the ferutinin preparation(s) were analyzed in triplicate by GC-MS. Separation was performed on an Autosystem XL (Perkin-Elmer) with a TurboMass detector fitted with a 30 m × 0.25 mm I.D. fused silica capillary column PE-5HT with a cross-linked 5% phenylpolydimethyl silicone stationary phase. The rate of helium flow through the

column was 1 ml/min. The injector worked at 225°C in split (1:50) mode. Chromatograms were registered using a linear temperature program regime ranging from 50°C to 300°C at a rate of 5°C/min. The electron impact mass spectra were obtained at 70 eV of ionization energy, a source temperature of 220°C and a detector temperature of 150°C. After integration, the fraction of ferutinin in the total ion current was calculated [33]. According to results of GC-MS analysis, the content of ferutinin in the preparation was 96 ± 2%.

A solution of C_{10}–C_{28} n-alkanes in n-hexane was separated under the conditions listed above. Linear temperature programmed retention indices (LTPRI) were calculated from the results of the separation of the n-alkanes and the methanol preparation solutions. The average LTPRI for ferutinin was 2778 ± 1 i.u. (n = 3). Figure 1 shows the mass spectrum of ferutinin.

For platelet experiments, ferutinin was prepared as a 25 mM stock solution in DMSO. The concentration of DMSO in the samples did not exceed 0.2%. In all experiments, an equivalent amount of DMSO was used as a control.

Blood collection, platelet detection in blood and platelet isolation

Blood was collected from 16 healthy male donors who were between 25 and 35 years of age and who had not taken any antiplatelet and/or antithrombotic drugs for at least 15 days before blood sampling. The blood from each subject was withdrawn by dripping from a 19-gauge needle placed in a peripheral vein into a tube containing an anticoagulant (0.105 M buffered sodium citrate). The final citrate : blood ratio was 1:9 v/v [22]. Care was taken to avoid undesirable artifactual platelet activation; for this purpose, donors rested for 20–30 min prior to the blood collection to minimize mental stress, and the first 0.5 ml withdrawn from the vein was discarded before the collection of blood used for further platelet functional assays. Flow cytometry measurements were performed within 2 h of blood collection [23].

To isolate platelets, blood was immediately centrifuged for 12 min at 190 × g to obtain platelet-rich plasma (PRP). Next, PRP was layered onto the top of BSA-Sepharose 2B gel columns prepared as previously described [34]. After the whole volume of the applied PRP had entered the gel, Tyrode's buffer was applied onto the column to elute the platelets.

Flow cytometry measurements

Preparation and staining were performed according to the standard Becton Dickinson Procedures for Flow Cytometric Analysis of Platelets, with slight modifications [6]. All flow cytometry experiments were carried out on whole blood, reducing the risk of undesirable artifactual platelet activation [23, 35]. Immediately after venipuncture, 10 µl aliquots of whole blood were added to 15 volumes of 1% CellFix, mixed, incubated at room temperature (RT) in the dark for 1 h, and then used to evaluate the extent of platelet activation in circulating blood.

The fluorescence emitted by 3000 platelets was measured using a LSR II Elite Flow Cytometer

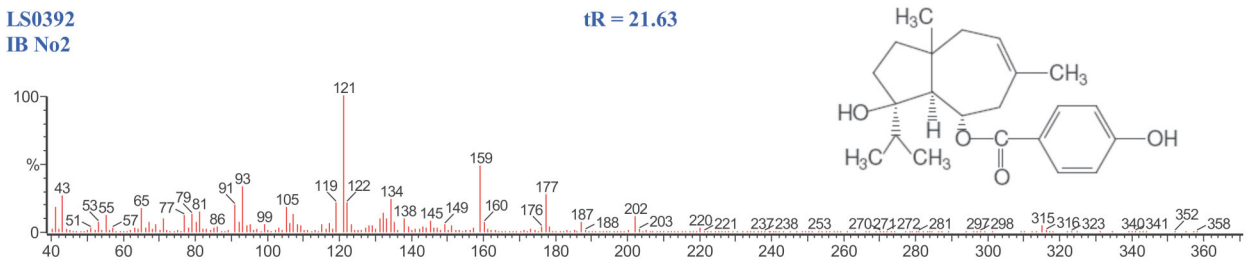

Fig. 1. Chemical structure and electron impact mass spectrum of ferutinin. Base Peak Intensities (≥ 1%) were: 40 (2); 41 (20); 42 (2); 43 (31); 44 (2); 45 (1); 50 (1); 51 (2); 52 (1); 53 (8); 54 (1); 55 (13); 56 (1); 57 (3); 62 (1); 63 (3); 64 (2); 65 (19); 66 (2); 67 (8); 68 (2); 69 (6); 70 (1); 71 (11); 72 (1); 77 (13); 78 (3); 79 (14); 80 (8); 81 (16); 82 (2); 83 (2); 84 (1); 85 (3); 86 (3); 89 (1); 91 (22); 92 (7); 93 (38); 94 (5); 95 (6); 96 (1); 97 (2); 99 (6); 100 (1); 102 (1); 103 (2); 104 (1); 105 (20); 106 (7); 107 (15); 108 (5); 109 (5); 110 (1); 111 (1); 113 (1); 114 (1); 115 (5); 116 (2); 117 (7); 118 (2); 119 (24); 121 (100); 122 (25); 123 (6); 124 (1); 125 (1); 126 (1); 127 (3); 128 (5); 129 (4); 130 (2); 131 (11); 132 (16); 133 (11); 134 (27); 135 (8); 136 (1); 138 (11); 139 (4); 140 (1); 141 (2); 142 (2); 143 (3); 144 (3); 145 (9); 146 (3); 147 (3); 148 (1); 149 (6); 150 (1); 151 (4); 152 (1); 153 (1); 155 (1); 156 (1); 157 (3); 159 (55); 160 (7); 161 (2); 162 (1); 163 (1); 173 (1); 174 (1); 176 (4); 177 (32); 178 (4); 185 (1); 187 (8); 188 (1); 200 (1); 202 (13); 203 (2); 205 (1); 220 (3); 315 (5); 316 (1); 358 (0.5)

equipped with a 13–20 mV solid state 488 nm Cohereut® Sapphire™ laser (Becton Dickinson). The fractions of the specific fluorescence-positive platelets were obtained after subtraction of non-specific fluorescence in the samples that had been labeled with only CD61/FITC. The percent of the antigen-positive platelets (i.e., antigen expression) reflects the abundance of the subpopulation of activated platelets, and the relative fluorescence intensity reflects the abundance of any given antigen on the platelet surface membranes. All flow cytometry measurements were adjusted for fluorescence on a daily basis for each set of measured samples using calibration beads (Becton Dickinson), to ensure that there was no considerable overlap among green, orange and red fluorescence.

The expression of the activated form of the fibrinogen receptor GPIIb-IIIa on the surface of the platelets was measured using PAC-1 antibodies conjugated to FITC. Briefly, 100 µl whole blood samples were incubated at RT with 50 µM ferutinin for 10 min. ADP (5 µM) was used as a positive control. Following the incubation, sample concentrations were adjusted to 1×10^7 platelets/ml with PBS containing 1 mM $MgCl_2$. After adjustment, 10 µl samples were supplemented with 2.5 µl PAC-1/FITC and 3 µl CD61/PerCP and incubated for another 20 min. The reaction was stopped with 1% CellFIX (150 µl) and samples were fixed for 1 h and diluted in CellWASH prior to measurements.

To assess the effects of ferutinin on the binding of fibrinogen (Fg) to human platelets, we performed a Fg binding assay according to a previously described method [31] with slight modifications. Human plasma-derived, Oregon Green® 488-labeled Fg was diluted with 0.1 M carbonate buffer (pH 8.3) to obtain a working solution at a concentration of 15 mg/ml. Blood samples (40 µl) were incubated for 10 min with this Fg solution at a final Fg concentration of 300 µg/ml in media containing 2.5 mM RGDS (negative control), 50 µM ferutinin or 5 µM ADP (positive control). Immediately after the incubation, the samples were fixed in 1% CellFix and incubated at RT for 1 h. Ten µl of each sample was labeled with 3 µl CD61/PerCP for 30 min at RT in the dark, followed by a 1 h incubation in 150 µl of 1% CellFIX. The samples were then resuspended in CellWash, and the fluorescences of Oregon Green®488 and PerCP were detected at 530 (band pass filter 30 nm, channel 1) and 695 nm (band pass filter 40 nm, channel 3), respectively. The binding of Fg was calculated based on the mean fluorescence intensity (MFI). The extent of specific Fg binding in all samples was estimated by subtracting out the Fg binding in control samples (those incubated with RGDS).

Measurement of intraplatelet Ca^{2+} using spectrofluorometry and flow cytometry

Mobilization of $[Ca^{2+}]_{in}$ in blood platelets was monitored using the following two assays: spectrofluorometry with Fura-2 on isolated platelets and flow cytometry with Fluo-3 on whole blood platelets. The first method supports direct calculation of $[Ca^{2+}]_i$ concentration ratios, while the second, although not perfectly linear, is not subject to artifactual platelet activation during platelet preparation [18].

To study the effect of ferutinin on intracellular Ca^{2+} using spectrofluorometry, aliquots of platelet-rich plasma were incubated with Fura-2 AM in Tyrode's buffer for 15 min at 37°C. The cell-permeable calcium-sensitive fluorescent probe Fura-2 AM was dissolved in DMSO to a final concentration of 1 mmol/l, divided into 50 µl aliquots and stored at –75°C until use.

Aliquots of platelet-rich plasma (4 ml each, at 2–3 $\times 10^8$ cells/ml) were incubated with Fura-2 AM at a final concentration of 4 µmol/l at 37°C in the dark for 15 min and then used for spectrofluorimetric determinations within 40 min. Incubation was terminated by gel-filtration of platelets on a 15 ml Sepharose 4B column equilibrated with calcium-free, magnesium-free Fura-Tyrode's buffer. One minute before the measurement, a 500-µl aliquot of Fura-2-loaded platelets was added to the cuvette and positioned in the water-heated holder, which contained 1 ml of calcium- and magnesium-free Tyrode's buffer. For estimation of the total $[Ca^{2+}]_{in}$, the sample was supplemented before measurement with Ca^{2+} to a final concentration of 1 mmol/l. To monitor the effect of ferutinin in the absence of Ca^{2+}, EGTA (rather than Ca^{2+}) was added to the sample to a final concentration of 1 mmol/l. Platelet activation was triggered by the addition of ADP (to a final concentration of 5 µmol/l) to the platelet medium. Measurement of the Fura-2-loaded platelets was performed under continuous stirring at 1000 rpm. The fluorescent calcium-free and complexed Fura-2 species were excited at 340 nm and 380 nm, respectively, using a 2.5-nm slit, and their emissions were recorded at 500 nm using a 5-nm slit. The measurements and analyses of $[Ca^{2+}]_{in}$ in platelets were performed on a LS50 spectrofluorometer (Perkin-Elmer, Beaconsfield, UK).

To study the effect of ferutinin on intracellular Ca^{2+} using flow cytometry, 500 µl of whole blood was incubated with 5 µM Fluo-3-AM and 150 µl anti-CD61/PerCP at RT in the dark for 30 min. At 5 min before the termination of the incubation, each sample was divided into 5 portions; four of them were incubated for an additional 5 min with 1 µM thapsigargin, 100 µM Gd^{3+}, 50 µM econazole (Eco) or 1 mM EGTA, respectively [36]. After this incubation, all of the samples were again divided, this time into two portions; half of the aliquots were incubated with ferutinin (50 µM) and the other half with ADP (5 µM). After the incubation, 10 µl of each sample was diluted with CellWASH and immediately measured by flow cytometry. A DMSO (diluent) control experiment was carried out simultaneously, and the calculations were performed relative to the Ca^{2+} levels in control platelets.

Monitoring of platelet aggregation

The ability of the platelets to aggregate was monitored in whole blood. Whole blood aggregation was monitored using a Whole Blood Aggregometer Chrono-Log 592 (Chrono-Log, Havertown, PA, USA), and the measurements were performed according to the Chrono-Log protocol [8]. Briefly, the whole blood was incubated for 15 min at RT. A 0.3 ml sample of whole blood was then diluted 1:1 with 0.85% saline, preincubated for 5 min at 37°C and supplemented with ferutinin to a final concentration of 50 µM. Platelet aggregation was monitored for 15 min. The value of impedance (Ω), which reflects the extent of maximal platelet aggregation (A_{max}), was used for further data analysis. Platelet reactivity was analyzed using "Platelet Aggregation Monitoring and Analysis" (PAMA) software [13].

Statistical analysis

The results are expressed as the arithmetic mean ± standard error of the mean (SE) or median (Me) and interquartile range (IQR: from lower quartile, Q1, to upper quartile, Q3). The data used for analysis are replicates of 2–4 single determinations of each studied sample. The differences were determined using either Student's *t* test or randomized block ANOVA for repeated measures for parametric data, or a Mann-Whitney U test for nonparametric data. Pearson's linear correlations were used to assess simple associations. Multiple regression methods were used for *post-hoc* data standardization (for the presence of confounding variables) and to determine partial effects (controlling for other accompanying variables), variable contributions (R^2_{corr}) and collinearity of variables. Spearman's (R_S) correlation was used to assess the associations between variables departing from the normal distribution [39].

Results

Effects of ferutinin and ADP on Ca^{2+} homeostasis in platelets from whole blood

Incubation of Fluo-3-stained whole blood platelets with 5 µM ADP resulted in an increased intracellular Ca^{2+} level in the whole blood platelets of 37.2 ± 3.0% in comparison to the level in resting platelets. Incubation with 50 µM ferutinin had a similar effect, raising intracellular Ca^{2+} by 25.4 ± 2.2% (Fig. 2). When extracellular Ca^{2+} was chelated with 1 mM EGTA, the ADP-stimulated increase in Ca^{2+} was decreased to 13.0 ± 3.0%, while that stimulated by ferutinin was almost completely abolished (0.4 ± 1.5% of the level in resting platelets (Fig. 2). With increased concentrations of EGTA (2 mM and 5 mM), the ADP-mediated increase in Ca^{2+} mobilization was slightly more reduced, but there was no further effect on the ferutinin-mediated change in Ca^{2+} (data not shown). Likewise, treatment of isolated platelets stained with Fura-2 with ferutinin (50 µM) resulted in a large increase in intracellular $[Ca^{2+}]_{in}$ (20.9 ± 1.5% of its control value, $p < 0.002$), and the chelating of extracellular Ca^{2+} with EGTA (1 mM) resulted in a dramatic reduction in the ferutinin-mediated increase in Ca^{2+} (3.9 ± 0.7% of the level in resting platelets, $p < 0.001$).

The ADP- and ferutinin-stimulated increases in Ca^{2+} transport across the plasma membrane were influenced to various degrees by different plasma membrane Ca^{2+} channel inhibitors. As shown in Figure 2, pre-treatment of cells with 100 µM Gd^{3+}, an inhibitor of voltage-dependent Ca^{2+} channels, did not noticeably change the elevation in Ca^{2+} caused by ferutinin, while the Ca^{2+} elevation in response to ADP in the presence of Gd^{3+} was considerably lower compared to that of the relevant control (Fig. 3). A similar effect

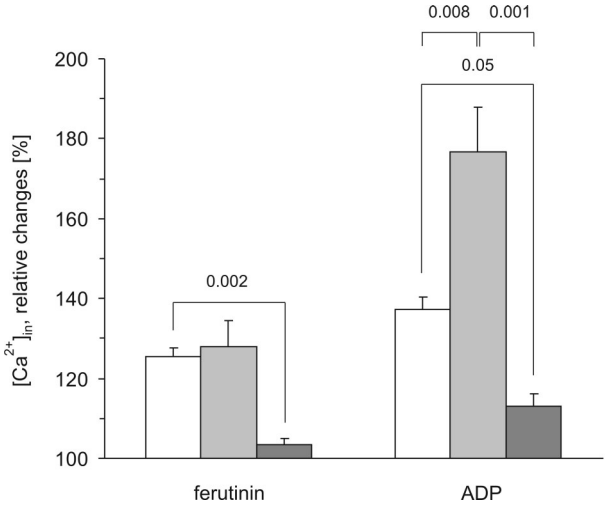

Fig. 2. Effects of thapsigargin and EGTA on ferutinin-mediated mobilization of intracellular Ca^{2+} in human platelets in whole blood. Data are expressed as the relative fluorescence intensities and presented as the means ± SEM; n = 9 (thapsigargin, TG) or n = 6 (EGTA) for ferutinin-activated platelets in the absence (open box) or presence of 1 μM TG (light grey shaded box) or 1 mM EGTA (dark grey shaded box). ADP (5 μM) was used as a positive control. The fluorescence intensities were normalized to the steady-state level before the application of the compounds (resting plt), which is designated as 100%. The significance of the differences was estimated using a one-tailed paired Student *t*-test with Bonferroni's correction for multiple comparisons. $p < 0.0003$, resting platelets *vs.* TG (data not shown); $p < 0.002$, resting platelets *vs.* ferutinin (data not shown)

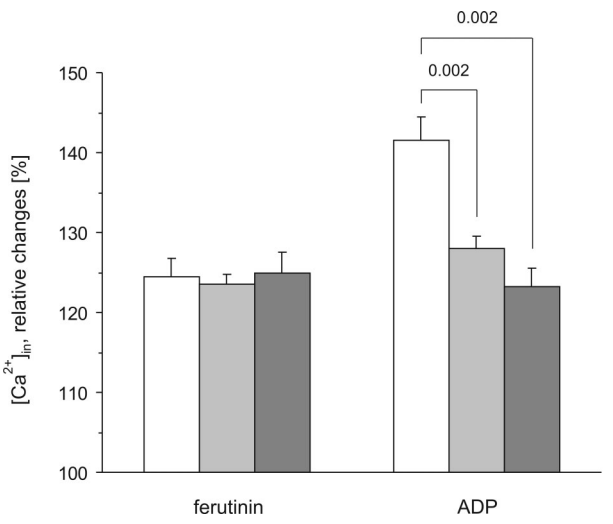

Fig. 3. The effects of inhibitors of plasma membrane Ca^{2+} channels on ferutinin-induced alterations in the intracellular free Ca^{2+} concentration in human platelets in whole blood. Data are expressed as the relative fluorescence intensities and presented as the means ± SEM; n = 5 for ferutinin-activated platelets in the absence (open box) or presence of either 100 μM Gd^{+3} (light grey shaded box) or 50 μM econazole (Eco, dark grey shaded box). ADP (5 μM) was used as a positive control. The fluorescence intensities were normalized to the steady-state level before the application of the compounds (resting plt), which is designated as 100%. The significance of the differences was estimated using a one-tailed paired Student *t*-test with Bonferroni's correction for multiple comparisons

was observed when an inhibitor of store-operated calcium channels, Eco (50 μM), was used. Again, the magnitudes of Ca^{2+} increase in response to ferutinin in the presence or absence of Eco were not statistically different, whereas the effect of ADP on Ca^{2+} mobilization in platelets was largely suppressed in the presence of Eco (Fig. 3).

Thapsigargin (TG), an inhibitor of dense tubular system-mediated Ca^{2+} sequestration, caused an increase in intracellular Ca^{2+} concentration in resting platelets of up to 13.4 ± 1.5% relative to the Ca^{2+} level in the control samples. When TG and ADP were used together, the mobilization of Ca^{2+} by ADP was greater than that stimulated by ADP alone (Fig. 2). There was no apparent difference in $[Ca^{2+}]_{in}$ elevation between the ferutinin and ferutinin plus TG conditions (Fig. 2).

Effects of ferutinin on the activation of fibrinogen (Fg) receptors and on platelet aggregation

The effect of ferutinin on the activation of Fg receptors in platelets in whole blood was significantly lower than the effect induced by ADP; the expression

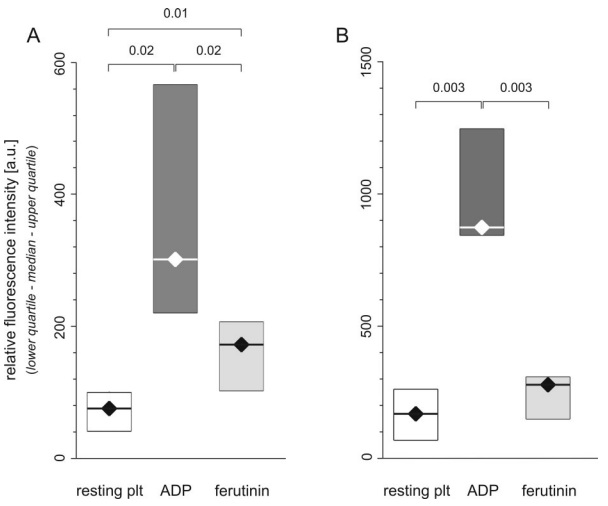

Fig. 4. Effects of ferutinin on the activation of fibrinogen receptor (GPIIb-IIIa) and fibrinogen binding in human platelets in whole blood. Data are expressed as the relative fluorescence intensity and presented as median (horizontal bar with a diamond sign) and lower-upper quartile range for resting platelets (open box) and platelets in the presence of ferutinin (shaded box). ADP (5 μM) was used as a positive control. The significance of the differences was estimated using a one-tailed signed rank Wilcoxon test with Bonferroni's correction for multiple comparisons

of the activated form of the GPIIb-IIIa complex was increased 2.2 fold upon ferutinin treatment and 5.6 fold upon ADP treatment (Fig. 4A).

In line with these observations were the results obtained for the binding of Fg to receptors on the surface of blood platelets in the presence of ferutinin. As shown in Fig. 4B, ferutinin also stimulated the binding of Fg to platelet receptors; its effect, however, was not statistically significant. In comparison, the receptor binding in the positive control (ADP) condition was 8-fold greater than that in resting platelets (Fig. 4B).

Overall, according to our data, ferutinin definitely increased cytosolic Ca^{2+} levels and stimulated activation of Fg receptors. Because of these results, we decided to investigate the effects of ferutinin on platelet aggregation. The effects of ferutinin on platelet aggregation were very weak (the mean ± SEM: 8.3 ± 0.6 a.u., $p < 0.001$) but significant, while the positive control, ADP, induced a massive platelet response (90.1 ± 2.5 a.u., $p < 0.0001$).

Discussion

Platelet aggregation may be caused by a variety of agonists, including thrombin, ADP and collagen. Despite such diversity in activation pathways and agonist structures, the general sequence of events during the initial response of platelets to all stimulatory factors is thought to be identical. Each agonist interacts with a specific membrane receptor, resulting in an increase in the intracellular concentration of calcium, a second messenger involved in cellular signaling [30]. Support for a role for Ca^{2+} ions in the process of platelet aggregation has been provided by experimental evidence showing that this process can also be triggered by Ca^{2+} ionophores [10, 14, 21]. The increase in intracellular Ca^{2+} induced by ADP, a weak natural agonist for platelets, is assumed to be mediated by both the release of Ca^{2+} from internal stores and Ca^{2+} influx from the external milieu.

In the present study, we showed that ferutinin induced an increase in $[Ca^{2+}]_{in}$ in platelets derived from human blood. In contrast to the events stimulated by ADP, this effect was almost completely blocked by the Ca^{2+} chelator EGTA and was not sensitive to the Voltage-Operated Calcium Channel (VOC) inhibitor Gd^{+3}, the Store-Operated Channel (SOC) inhibitor Eco, or the SR/ER Ca^{2+}-ATPase inhibitor Tg.

These experimental data encouraged us to speculate that, in contrast to ADP, which most likely increases $[Ca^{2+}]_{in}$ *via* the activation of platelet plasma membrane Ca^{2+} channels and the release of Ca^{2+} from intracellular stores, ferutinin may increase Ca^{2+} levels because of its Ca^{2+}-ionophoretic properties. Ferutinin is considered an electrogenic Ca^{2+} ionophore [2, 38], and its effects on calcium homeostasis differ from those of classical neutral Ca^{2+} ionophores like A23187 or ionomycin [1]. Neutral ionophores are known to dissipate Ca^{2+} gradients in a cell when added after the addition of TG, leading to increased $[Ca^{2+}]_c$ as a result of the release of Ca^{2+} from mitochondria. It has been previously shown in hepatocytes and neurons that unlike neutral Ca^{2+} ionophores, ferutinin does not change the $[Ca^{2+}]_c$ levels in cells that have been preincubated with TG [1]. It is hypothesized that ferutinin acts as an electrogenic uniporter, thereby inducing the accumulation of Ca^{2+} released from the ER in response to TG in the mitochondria. This may be one of the reasons why we did not observe alterations in $[Ca^{2+}]_c$ following preincubation with TG in our experiments. Moreover, ferutinin is also known to specifically interact with estrogen receptors, thus mimicking the effects of classic estrogens [4, 16]. However, the influence of ferutinin on intraplatelet Ca^{2+} content in blood platelets differs from the effects of steroids and phytosteroids. It has been previously shown that 17β-estradiol and progesterone do not alter the intracellular levels of Ca^{2+} in resting platelets [23, 24, 26]; however, these two estrogens do reduce the $[Ca^{2+}]_{in}$ elevation induced by vasopressin in human platelets [27]. Likewise, phytoestrogens and a synthetic estrogen agonist, diethylstilbestrol, have been shown to inhibit thrombin-induced Ca^{2+} influx into human platelets [11, 37].

Because we have established that ferutinin, unlike classic estrogens, increases intraplatelet Ca^{2+} levels, we further investigated its influence on Ca^{2+}-dependent processes in blood platelets, such as the activation of Fg receptors, Fg binding and platelet aggregation.

Ferutinin had very little to no activity in terms of 'priming' platelets for aggregation. Ferutinin very slightly stimulated the expression of the active GPIIb-IIIa complex and whole blood platelet aggregation, and it had no significant influence on the binding of Fg to platelets. Despite a significant increase in

the intraplatelet calcium concentration, the effect of ferutinin on platelet aggregation was negligible compared with that of a typical platelet agonist, ADP. Thus, the effects of these two agents on $[Ca^{+2}]_{in}$ in blood platelets were completely unrelated to their variable influences on platelet aggregation. This part of our data appear to contradict other data showing effects of platelet agonists and calcium ionophores on platelet aggregation [7, 10, 28]. The absence of strong effects on aggregation, expression of the active form of the Fg receptor, and the binding of Fg to platelets are difficult to reconcile with ferutinin's role as an ionophore. Previously, it was shown that the Ca^{2+} concentration required for platelet aggregation and platelet shape change induced by ionomycin is higher when cells are treated with ADP [14]. It is possible that in this particular case, the ferutinin-mediated increase in $[Ca^{2+}]_{in}$ is not sufficient to trigger the cellular signaling events that lead to platelet aggregation. This might be due to the redistribution of the accumulated Ca^{2+} ions into mitochondria, which could lead to a decrease in mitochondrial potential, the opening of mitochondrial membrane permeability transition pores and, finally, the collapse of cellular energy storage systems required for efficient platelet aggregation. It has been shown that ferutinin-mediated Ca^{2+} transfer in Jurkat cells results in mitochondrial depolarization that is blocked by cyclosporin A [20]. Also, not all agents with recognized Ca^{2+} ionophoric properties induce platelet aggregation; for instance, lasalocid and its derivatives are known to deplete intraplatelet serotonin storage pools without leading to obvious platelet aggregation [21].

Recently, it has been demonstrated that estrogens exert rapid, non-genomic effects in various type of cells [12]; these effects include changes in Ca^{2+} homeostasis (stimulation/inhibition of Ca^{2+} influx or depletion of intracellular calcium stores), activation of adenylate cyclase and nitric oxide synthase and stimulation of mitogen-activated protein kinases [5, 12, 19]. All of these effects have been proposed to be mediated by the interaction of the hormones with plasma membrane estrogen receptors, and, recently, the estrogen receptors ERa and ERb have been found at the platelet membrane [17, 26].

On the other hand, much of the available literature concerning the influence of estrogens on the intracellular levels of second messengers and their effects on aggregation of platelets appears contradictory. In a number of studies, it has been shown that 17β-estradiol does not alter the intracellular levels of second messengers like cAMP or cGMP and does not lead to any detectable aggregation of platelets but does modulate the effects of other agonists on blood platelets [24, 25]. Nakano et al. found that 17β-estradiol inhibited both ADP- and thrombin-induced platelet aggregation by increasing the level of nitric oxide (NO), a potent inhibitor of platelet aggregation that contributes to the elevation of cGMP but not cAMP [25]. Other studies have also shown that 17β-estradiol potentiates thrombin-induced platelet aggregation mediated by ER_{β} receptors *via* mechanisms involving the activation of the tyrosine kinases Src and Pyk2 [24, 29]. The rather confusing array of data on 17β-estradiol presented above may be explained by variations in methodology, including differences in the quality of platelet preparation, the concentrations of the hormones, and/or the duration of pre-treatment with hormones. For instance, it has been shown that the effects of 17β-estradiol depends on the sensitivity of the platelets to aggregating agents. Estradiol increased epinephrine- and ADP-stimulated aggregation in hyperreactive platelets but also inhibited platelet aggregation in hypoaggregative cells [3].

Based on the above reasoning, we argue that the Ca^{2+}-elevating effect of ferutinin resulting from ionophoric activity may be responsible for stimulation of Ca^{2+}-dependent NO synthase and increased NO levels. In turn, NO, either directly or indirectly *via* the generation of cGMP or cAMP, can stimulate the reaccumulation of Ca^{2+} into stores, thus reducing the $[Ca^{2+}]_{in}$ in platelets, and hampering platelet aggregation in the presence of ferutinin alone. The possibility that NO synthase may be activated by ferutinin in platelets is supported by the previously reported finding that ferutinin at high concentrations (>300 μM) stimulates NO synthase in the median eminence of the rat brain [9]. We cannot, however, exclude the possibility that the ameliorative effect of ferutinin on blood platelets is also mediated by increases in platelet cAMP level through its interaction with estrogen receptors that are coupled to adenylate cyclase *via* plasma membrane G-proteins.

In conclusion, although the detailed molecular mechanism(s) of the various aforementioned effects of ferutinin are not yet clear and remain a topic for future investigations, these interesting properties of ferutinin increase its therapeutic potential. Because ferutinin is a plant estrogen with low toxicity and is likely to be used in gynecological therapies in the fu-

ture, it is important to note its lack of pro-aggregatory properties, which help categorize it as a non-hazardous agent that is certainly worthy of further study.

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