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ORIGINAL ARTICLE

Flavonoid inhibition of platelet procoagulant activity and phosphoinositide synthesis

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Summary. Dietary flavonoids are known for their antiplatelet activity resulting in cardiovascular protection. Phosphatidylinositol 4,5-bisphosphate (PIP_2) was previously reported to play a direct role in phosphatidylserine (PS) exposure, as a Ca^{2+} target. Thrombin formation and platelet procoagulant activity are dependent on PS exposure. As flavonoids can inhibit phosphoinositide (PPI) kinases, we examined whether changes in PPI metabolism in flavonoid-treated platelets could be involved in their antiplatelet effects. Treatment with the flavonoids quercetin or catechin reduced PS exposure, thrombin formation, PIP_2 level and resynthesis after platelet activation with collagen, thrombin or calcium ionophore. Flavonoids also prevented $[Ca^{2+}]_i$ increase induced by collagen, but not by the ionophore. The ability of flavonoids to decrease PS exposure induced by ionophore treatment could result from the diminution of PIP_2 levels, whereas PS exposure induced by collagen could also be diminished by flavonoids' effects on calcium signaling dependent on PIP_2 hydrolysis. These data favor a role for PIP_2 in the antiplatelet effects of flavonoids.

Keywords: flavonoid, phosphatidylserine, PIP_2, platelets.

Introduction

Several epidemiological studies have shown an inverse relationship between consumption of natural flavonoids, such as quercetin and catechin, and the frequency of coronary heart disease and stroke [1,2]. These common components of the diet possess antioxidant properties, manifested by their ability to inhibit the enzymatic generation of free radicals, to trap free radicals, and to block the oxidation of cellular membrane constituents and plasma components [3,4]. In addition, flavonoids inhibit synthesis of leukotrienes in neutrophils [5], promote nitric oxide production by vascular endothelium [6], arrest tumor growth, and inhibit carcinogenesis [3,7]. Flavonoids may also affect the activity and concentration of plasma hemostasis factors such as fibrinogen, factor (F)VII, and plasminogen [8]. Additionally, quercetin inhibits angiotensin II-induced hypertrophy of vascular smooth muscle, partly by its inhibitory effect on Shc- and Phosphatidylinositol (PI) 3-kinase-dependent c-Jun N-terminal kinase activation [9]. A number of protein serine/threonine kinases (e.g. AMPK, CK2, MAPKAP-K1/RSK2 and S6K1) are also inhibited by quercetin with a potency similar to that for PI-3 kinase inhibition [10]. Generation of PI-3 kinase lipid products is required for platelet spreading as well as for irreversible platelet aggregation [11].

Flavonoids are beneficial in cardiovascular systems, primarily by inhibiting platelet function. Several *in vitro* studies show that these components inhibit platelet aggregation [12–14], a major process contributing to both the development of atherosclerosis and acute platelet thrombus formation followed by embolism-producing cyclic flow reduction in stenosed arteries. Flavonoid antiplatelet effects may be attributed to inhibition of thromboxane formation, thromboxane receptor antagonism [12], blunting hydrogen peroxide production, or inhibition of phospholipase C [13]. Inhibition of Ca^{2+} influx, internal Ca^{2+} release, protein kinase C (PKC) activation, and actin polymerization [5,14] can also contribute to their antiplatelet effects. A potential limitation of these investigations is that the flavonoid concentrations used to observe these effects (>5–1000 μM) were much higher than those prevailing in plasma (approximately 1.5 μM), even after diets supplemented with flavonoids. Consequently, some authors have questioned the antiplatelet properties of these components *in vivo* [8].

Platelets are essential for the catalysis of two reactions of the blood coagulation cascade: the activation of FX to FXa and the conversion of prothrombin to thrombin by a complex of FXa,

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FVa and Ca^{2+} [15]. Activated platelets provide a catalytic surface for these reactions, as a result of the externalization of phosphatidylserine (PS) which, in conjunction with other phospholipids is redistributed across the plasma membrane (phospholipid scrambling). PS exposure strongly correlates with generation of the procoagulant activity of platelets [16]. The mechanism of PS exposure is not fully understood. A 37-kDa protein, located in plasma membrane and possessing scrambling activity (scramblase), has been isolated from platelets and erythrocytes, cloned and sequenced [17]. A role for phosphatidylinositol 4,5-bisphosphate (PIP_2) in phospholipid scrambling in red cells [18–21] and in phosphatidylserine exposure in platelets [22] has been proposed. The mechanism responsible for this PIP_2 function, although controversial [23], would involve interaction between Ca^{2+} or other polycationic effectors and PIP_2 [19,20,22]. Since flavonoids can inhibit phosphatidylinositol 4-phosphate (PIP) and PIP_2 synthesis in tumors [3,7] and chromaffin cells [24], we hypothesized that a change in phosphoinositide (PPI) metabolism in flavonoid-treated platelets could be a potential mechanism to explain their antiplatelet effects. In this paper, we show that flavonoid treatment reduced the concentration of PIP_2 and inhibited PIP_2 resynthesis in platelets stimulated with collagen, thrombin or calcium ionophore. These changes in PPI metabolism correlated with inhibition of various platelet functions, including PS exposure, supporting the concept that PIP_2 is involved in the antiprocoagulant activity of flavonoids.

Materials and methods

Isolation of human platelets

Blood from healthy volunteers was collected in acid-citrate dextrose. Platelet-rich plasma (PRP) obtained after centrifugation (15 min, 110 *g*) at room temperature was supplemented with apyrase (0.5 U mL^{-1}). Platelets were sedimented by centrifugation (10 min, 1000 ×*g*), suspended in buffer A (139 mM NaCl, 2.8 mM KCl, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 8.9 mM $NaHCO_3$, 10 mM HEPES, 5.6 mM glucose, 0.3% albumin, pH 7.35) and filtered on a 50-mL column of Sepharose 2B to obtain gel-filtered platelets (GFP). Quercetin or catechin (Sigma, St Louis, MO, USA; stock solution in DMSO) was added to PRP or GFP suspensions (2×10^8 mL^{-1}) and incubated for 30–40 min at 37 °C before platelet stimulation.

Platelet aggregation

Platelet suspensions in buffer A containing 2 mM $CaCl_2$ were placed into prewarmed cuvettes (37 °C) of a Chronolog Lumi-Aggregometer (Chrono-Log Corp., Havertown, PA, USA). Aggregation was initiated by adding 20 mg mL^{-1} collagen (Chrono-Log Corp.) or 2 μM calcium ionophore (A23187; Sigma) and stirring at 1000 r.p.m. Changes in light transmission were recorded for 10 min, using a PowerLab/200 instrument and MacLab Chart program Version 3.2.

Platelet secretion

Platelet secretion was measured by a ^{14}C-5-hydroxytryptamine (^{14}C-5-HT) release assay, as described by Rogers *et al.* [25]. Briefly, purified platelets were preincubated with ^{14}C-5-HT (Amersham Biosciences, Little Chalfont, Bucks, UK; spec. act. 50–62 mCi $mmol^{-1}$) at 0.05 mCi mL^{-1} of platelet suspension for 60 min at 37 °C. Following incubation, platelets were treated with flavonoids and then activated with 2 μM A23187 or 20 μg mL^{-1} collagen for 5 min without stirring. After addition of acetylsalicylic acid (15 mM) to stop activation, samples were centrifuged at 6000 *g* for 5 min. Aliquots (50 μL) of the supernatants and of the total suspensions were counted in a LS 6500 scintillation counter (Beckman Coulter Inc, Fullerton, CA, USA). Results are expressed as the percentage of released ^{14}C-5-HT.

Measurement of intracellular $[Ca^{2+}]_i$

Cytosolic free Ca^{2+} concentration was measured in platelets loaded with Fura-2 AM (Sigma), according to Pollock *et al.* [26]. Changes in fluorescence were monitored with an SLM-Aminco MC200 fluorimeter set at 340 nm excitation and 510 nm emission wavelengths. The $[Ca^{2+}]_i$ level was calculated using the general formula $[Ca^{2+}]_i = K_d(F - F_{min}/F_{max} - F)$ in which K_d is the dissociation constant of Fura-2 for Ca^{2+} binding (224 nM), and F the fluorescence intensity of the sample. F_{max} was determined after lysing the cells with digitonin and F_{min} after adjusting the pH of the lyzed cells to 8.5, followed by the addition of EGTA.

PS exposure

PS exposure on the outer platelet surface was determined from the binding of FITC-labeled annexin V (AV–FITC), as described by Andersen *et al.* [27]. Platelets preincubated with or without flavonoids in buffer A containing 3 mM $CaCl_2$ were activated at 37 °C for 20 min, without stirring, by 2 μM A23187, 20 μg mL^{-1} collagen, 10 μM ADP (Sigma), 50 μM TRAF (thrombin receptor activated fragment SFLLRN from Bachem) or by a combination of 20 μg mL^{-1} collagen and 1 U mL^{-1} human thrombin (Sigma). After addition of AV–FITC (Sigma) for 20 min at room temperature, cells were rinsed and centrifuged (3 min, 1000 *g*) to remove unbound AV–FITC. Bound AV–FITC was released from remnant platelets using a buffer containing 50 mM EDTA, and its amount was estimated by fluorimetry ($I_{ex} = 495$ nm, $I_{em} = 519$ nm). PS exposure was expressed as fold increase in bound AV–FITC relative to that in unstimulated platelets. Cell lysis occurring during this assay was determined by LDH activity (Sigma kit) in the supernatant of activated platelets. Depending on the experimental conditions, lysis amounted to 1–3% of total cells.

Preparation of large unilamellar vesicles (LUVs)

A dry lipid film of phosphatidylcholine (PC) (1.66 nmol) or PC/PS (50% each) was hydrated with 0.5 mL buffer B (140 mM

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NaCl, 10 mM HEPES, 0.1 mM EGTA, pH 7.35) and vortexed. The mixture was freeze–thawed five times and then extruded through polycarbonate filters (200 nm) five times. LUV size (hydrodynamic diameter) was determined by dynamic light scattering using a DynaPro 99 instrument. LUV phospholipid composition was determined by thin layer chromatography (TLC) analysis using silica plates (Merck KGaA, Darmstadt, Germany) developed with chloroform : methanol : acetic acid : H_2O (77 : 49 : 10 : 6 by vol.) [28].

Measurement of thrombin formation in PRP and in platelet-poor plasma (PPP) after Ca^{2+} loading

Platelet procoagulant activity was determined as described previously [29], by measuring thrombin formation in Ca^{2+}-loaded PRP as well as in PPP in which platelets were replaced by LUVs. Experiments with PS-containing LUVs were designed to determine whether flavonoids could inhibit prothrombinase activity by affecting plasma factors (such as Xa and Va) by a mechanism not dependent on preventing exposure of PS in platelet plasma membranes. PRP and PPP were prepared by centrifugation and when required, preincubated with flavonoids. PRP was activated with collagen (20 $\mu g\ mL^{-1}$) or A23187 (2 μM) for 10 min. PPP was preincubated with LUVs (with or without PS) for the same period. The reaction was started by adding prewarmed $CaCl_2$ to all samples. At various time points, 100-μL aliquots of plasma were removed and mixed with 100 μL PBS containing 20 mM EDTA to stop prothrombinase activity. Thrombin formation was estimated from cleavage of the synthetic peptide tosyl-glycyl-arginine−4-nitroanilide (Chromozym TH; Roche Diagnostics GmbH, Mannheim, Germany), which mimics the N terminus of the α-chain of human fibrinogen, by following optical density changes at 405 nm.

PPI metabolism in ^{32}P-labeled platelets

GFP, prepared in phosphate-free buffer A, were pelleted by centrifugation, resuspended at a concentration of $8 \times 10^8\ mL^{-1}$, treated with prostaglandin E $(PGE)_1$ (1 μM) and incubated for 90 min at 37 °C, with 0.5 mCi of ^{32}P-orthophosphoric acid-mL^{-1} of platelet suspension. After centrifugation, platelets were resuspended and adjusted to a final concentration of $2 \times 10^8\ mL^{-1}$ in phosphate-free buffer A containing 2 mM $CaCl_2$. Depending on the experimental conditions, flavonoids were added before or after the labeling process, for a 30-min incubation period. Activation of platelets was performed at 37 °C on 1-mL aliquots, without stirring, by the addition of 2 μM A23187, 20 μg collagen or 1 U mL^{-1} thrombin. The reaction was terminated by adding 3.75 mL chloroform : methanol : conc. HCl (20 : 40 : 1). Lipid extraction was continued for 1 h at room temperature. Phase separation was obtained by addition of 1.25 mL each of chloroform and water. Samples were then centrifuged (1000 × *g*, 10 min, 4 °C), and the chloroform phase was withdrawn. Before TLC analysis, lipids were dried under a stream of N_2, suspended in chloroform : methanol (2 : 1), and separated on boric acid-impregnated silica TLC plates (Merck) using the solvent 1-propylacetate : 2-propanol : absolute ethanol : 6% aqueous ammonia (3 : 9 : 3 : 9, by vol) [30]. ^{32}P-labeled $PI(4,5)P_2$, PI(4)P, and phosphatidic acid (PA) standards for TLC were prepared by *in vitro* incubation of human erythrocytes with ^{32}P-orthophosphoric acid, essentially as previously described [31].

Statistical analysis

Data are reported as means ± SD from three to 10 experiments. Differences between means were evaluated by Student's *t*-test, with $P < 0.05$ being taken as the level of significance.

Results

Platelet aggregation

The effect of flavonoids on platelet aggregation was determined after stimulation with either collagen or calcium ionophore.

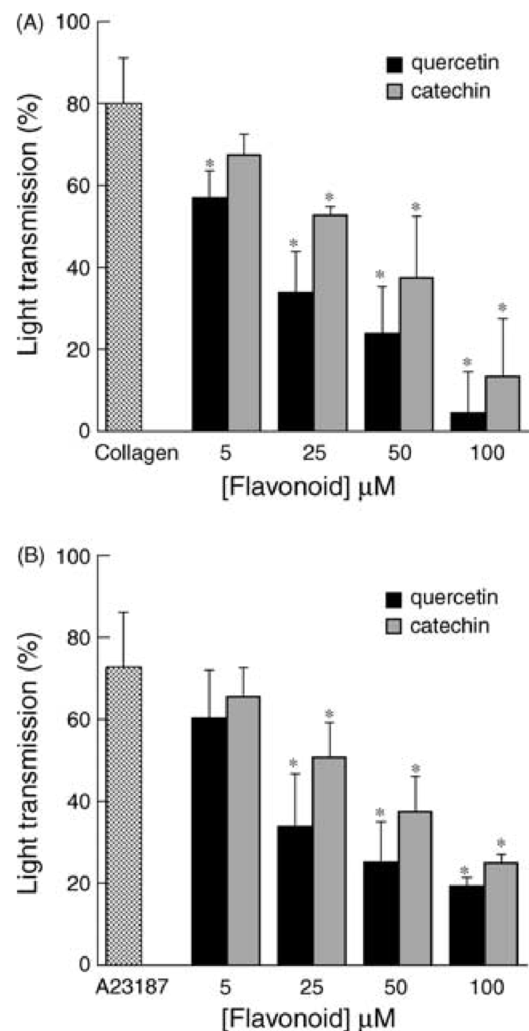

Fig. 1. Effect of flavonoids on platelet aggregation. Total platelet aggregation was measured from the slope in the change of light transmission after 10 min addition of collagen (A) or A23187 (B) to human platelets pretreated for 40 min with various concentrations of quercetin or catechin. Data are means ± SD of five experiments. *Significantly different from control.

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(Fig. 1A,B). Aggregation induced by each stimulus was inhibited dose-dependently by quercetin or catechin. Quercetin was more potent than catechin and caused significant inhibition at 5 μM and 25 μM, respectively, when aggregation was induced by collagen. Aggregation induced by calcium ionophore was inhibited at 25 μM of either quercetin or catechin. Neither wortmannin (10 nM) nor LY294002 (10 μM) had similar inhibitory effects, nor did these specific PI 3-kinase inhibitors prevent the effect of the flavonoids (data not shown).

Platelet secretion

The effect of flavonoids on α-dense granule secretion was determined from the release of ^{14}C-5-HT after stimulation of platelets with either collagen or calcium ionophore (Fig. 2A,B). Quercetin was more potent than catechin to inhibit granule secretion. Significant inhibition of platelet secretion induced by collagen was observed with 5 μM quercetin and 25 μM catechin. After stimulation with A23187, inhibition was significant with 25 μM quercetin and 100 μM catechin.

Platelet $[Ca^{2+}]_i$

Calcium concentration was measured in Fura 2 AM-loaded platelets after stimulation with either collagen or calcium ionophore (Fig. 3A,B). After collagen activation, quercetin or catechin, from 25 μM, significantly inhibited the increase in platelet $[Ca^{2+}]_i$ (Fig. 3A), whereas flavonoids, at any concentration, did not affect the increase in $[Ca^{2+}]_i$ induced by A23187 (Fig. 3B).

PS exposure

The effects of flavonoids on PS exposure in platelets, shown in Fig. 4, were assessed from the binding of FITC–annexin V, after activation of platelets with various stimuli. Flavonoid by

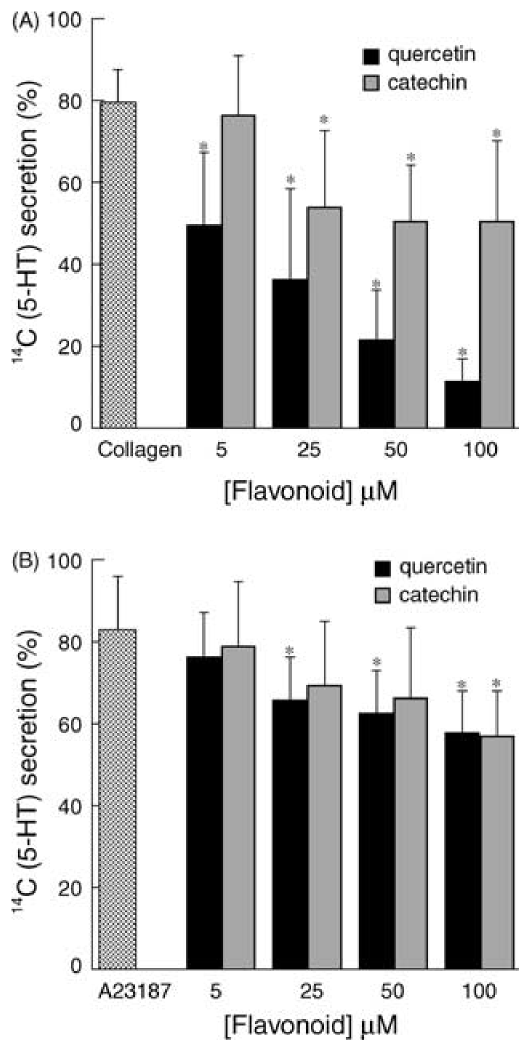

Fig. 2. Effect of flavonoids on platelet secretion. Platelets pretreated for 40 min with various concentrations of quercetin or catechin were stimulated for 5 min with collagen (A) or A23187 (B). Data are means ± SD of five to seven experiments. *Significantly different from control.

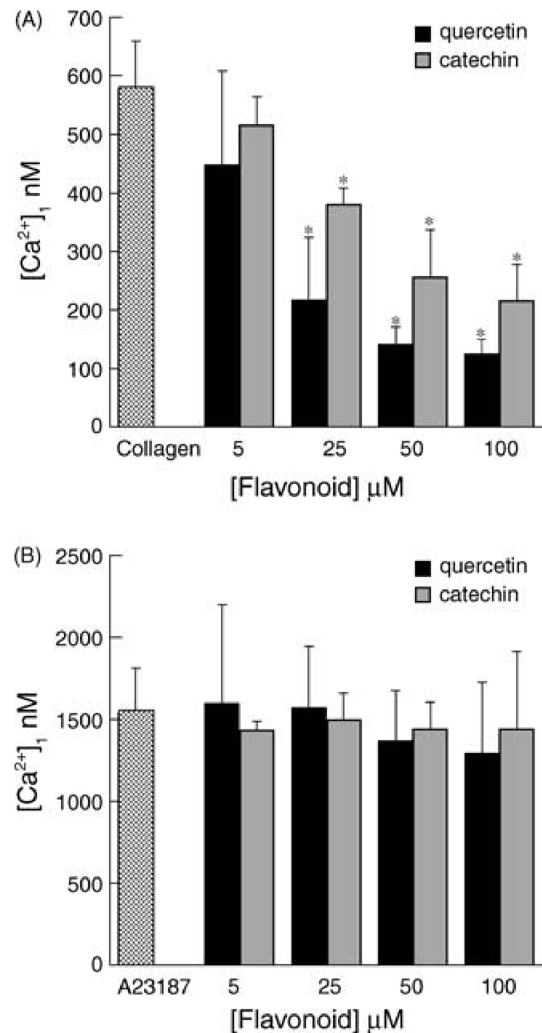

Fig. 3. Effect of flavonoids on platelet $[Ca^{2+}]_i$ concentration. Fura 2 AM-loaded platelets, pretreated for 40 min with various concentrations of quercetin or catechin, were stimulated with 20 μg mL^{-1} of collagen (A) or 2 μM of A23187 (B). Data are means ± SD of four experiments. *Significantly different from control.

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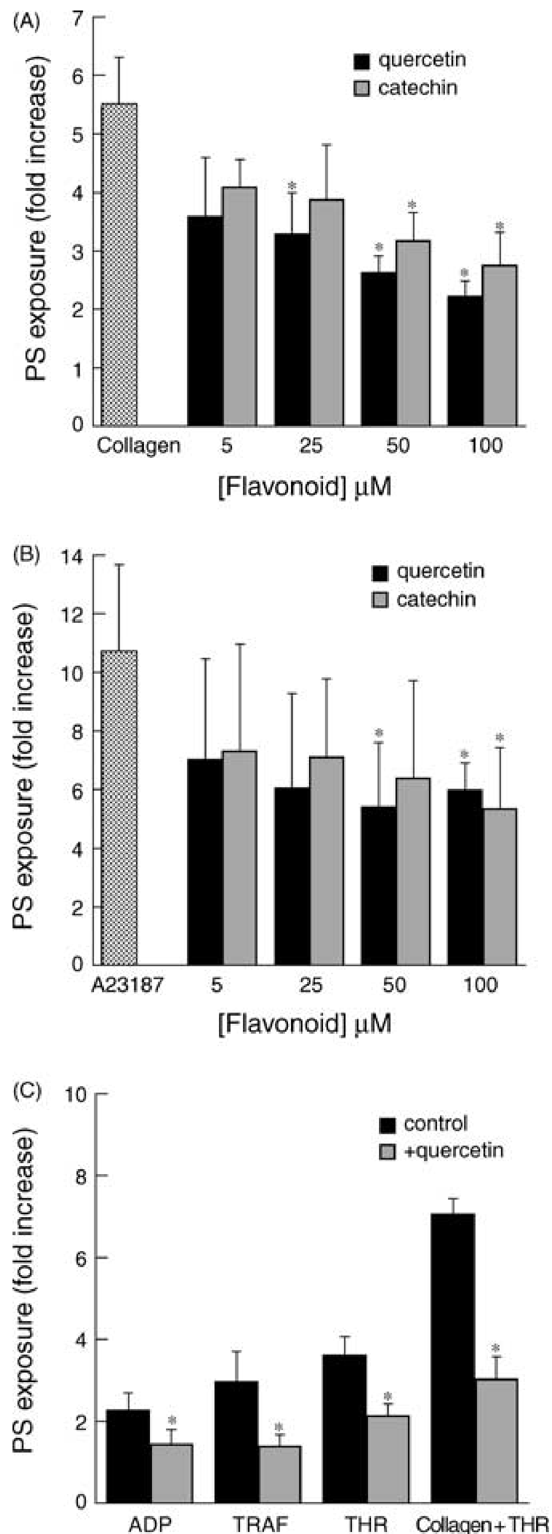
themselves had no effect on the level of PS exposure in resting cells. Both quercetin and catechin, from concentrations of 25 μM and 50 μM, respectively, significantly inhibited PS exposure induced by collagen (Fig. 4A). Likewise, PS exposure induced by A23187 was inhibited by flavonoids, but the effect was weaker than that observed with collagen and was significant from 50 μM for quercetin and from 100 μM for catechin (Fig. 4B). Quercetin (100 μM) also inhibited PS exposure induced by other platelet agonists (Fig. 4C), and maintained a low level of PS exposure independently of the absolute level induced by different agonists. It was recently reported that quercetin and catechin synergistically inhibit platelet function [13]. In our study, we did not observe any synergistic effect of a combination of 25 μM catechin and 5 μM quercetin on PS exposure induced by 20 μg mL^{-1} collagen (data not shown).



Since platelet activation leads to shedding of vesicles, and PS exposure was measured in remnant cells, it was important to determine whether the inhibition induced by flavonoids could result from an increase in microparticle shedding. Light scattering measurements and phospholipid assays, to estimate the extent of microparticle production, indicated that quercetin (100 μM) did not increase vesicle shedding induced by collagen or ionophore (data not shown). Furthermore, we have checked that the inhibitory effect of quercetin and catechin on platelet aggregation and PS exposure did not result from the inhibition of PI 3-kinase, which is involved in some responses of activated platelets [11]. Indeed, the effects of the flavonoids were not altered by prior treatment with wortmannin (10 nM) or LY294002 (10 μM) (data not shown). To investigate whether the inhibition of PS exposure induced by flavonoids could be related to their previously reported antioxidant properties, we tested the effect of other antioxidants on PS exposure induced by thrombin. Neither tocopherol (200 μM) nor catalase (1000 U mL^{-1}) inhibited PS exposure (data not shown), suggesting an oxidation process is not involved in the mechanism of PS exposure.

Effects of flavonoids on prothrombinase activation in Ca^{2+}-enriched plasma

In PRP activated with collagen or A23187, thrombin production was linear between 5 and 15 min after addition of Ca^{2+}, and decreased at longer incubation times (data not shown). Preincubation of PRP with flavonoids decreased the thrombin production detected after 15 min in a concentration-dependent fashion (Fig. 5). Thrombin production in PRP after collagen stimulation was significantly inhibited when platelets were preincubated with 50 μM or 100 μM quercetin or catechin (Fig. 5A). Flavonoids were more effective at inhibiting transformation of prothrombin to thrombin in PRP stimulated with collagen than with calcium ionophore (Fig. 5B). Because

Fig. 4. Effect of flavonoids on phosphatidylserine (PS) exposure. Platelet suspensions were pretreated for 40 min with flavonoids before activation with different agonists for 20 min. The amount of cell-bound FITC–annexin V (PS exposure) in treated samples was expressed as fold increase relative to bound annexin V in control samples. (A) Dose-dependent inhibition of collagen-induced PS exposure by quercetin or catechin. (B) Dose-dependent inhibition of calcium ionophore-induced PS exposure by quercetin or catechin. (C) Effect of 100 μM quercetin on PS exposure induced by different platelet agonists. Data are means ± SD of three to four experiments. *Significantly different from control.

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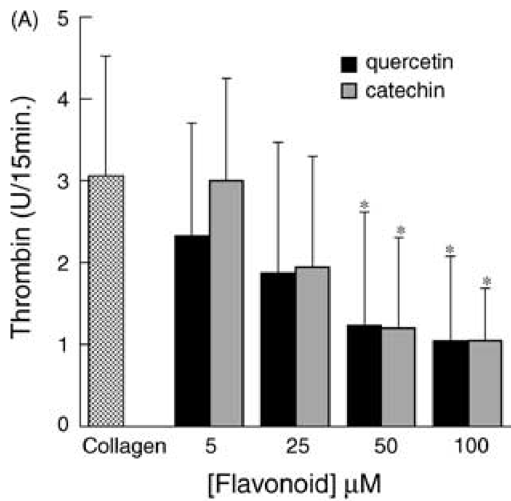
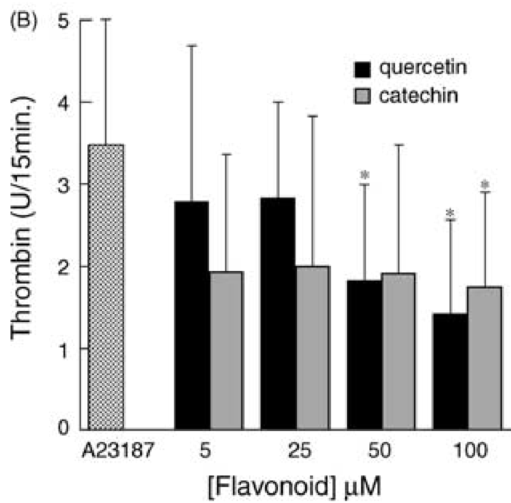




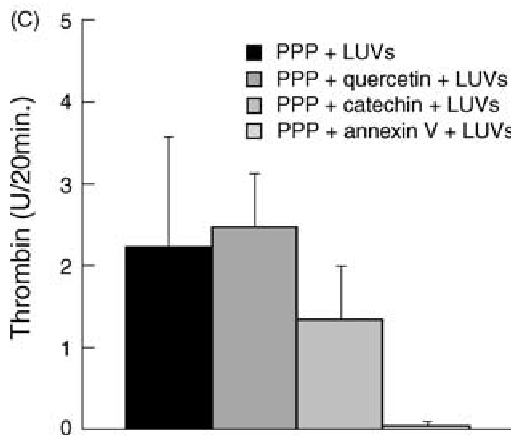
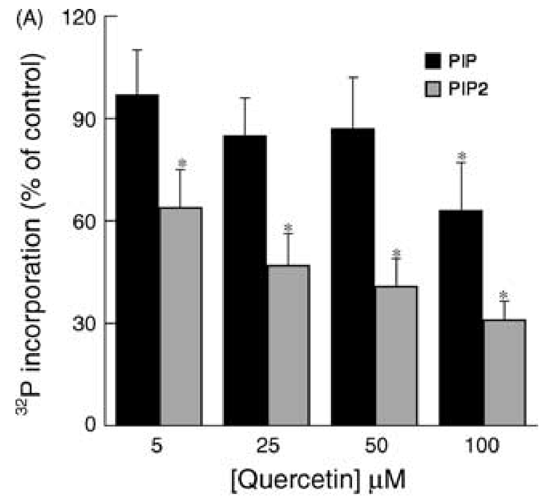
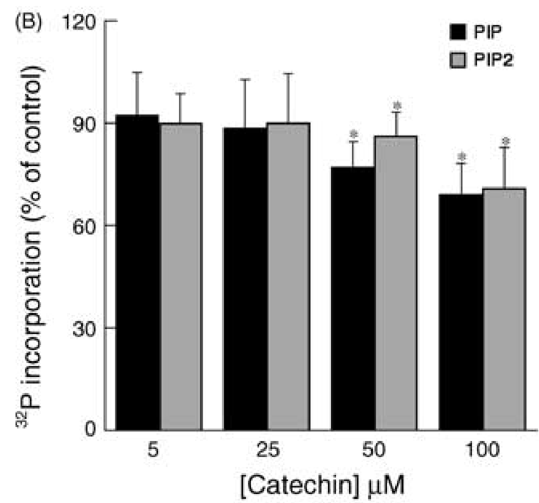


Fig. 5. Effect of flavonoids on thrombin generation. Thrombin formation in platelet-rich plasma (PRP) preincubated for 40 min with different concentrations of quercetin or catechin was measured after activation with collagen (A) or calcium ionophore (B) for 10 min. Thrombin formation in platelet-poor plasma (PPP) (C) preincubated with flavonoids (100 µM) or annexin V (100 nM) for 40 min was measured after addition of phosphatidylcholine phosphatidylserine (PS/PC) large unilamellar vesicles (LUVs) and Ca^{2+} for 10 min and 20 min, respectively. Data are means ± SD of five to 10 experiments. *Significantly different from control.





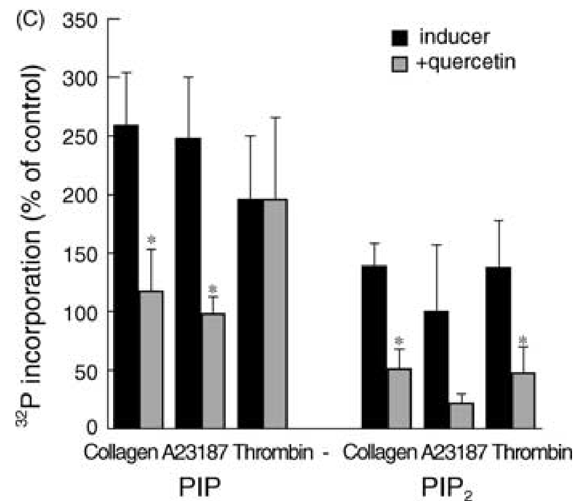


Fig. 6. Effect of quercetin and catechin on phosphatidylinositol 4-phosphate (PIP) and on phosphatidylinositol 4,5-bisphosphate (PIP_2) metabolism. After preincubation for 20 min with different concentrations of quercetin (A) or catechin (B), platelets were labeled with ^{32}P-orthophosphate for 90 min. Platelets were ^{32}P-labeled for 90 min, washed, treated with or without 100 µM quercetin for 30 min and stimulated for 10 min with collagen, A23187 or thrombin (C). Data are means ± SD of three to five experiments. *Significantly different from corresponding control.

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prothrombinase activity is not only dependent on PS exposure, but also on FXa and FVa activation, its inhibition by flavonoids might also be mediated through a direct effect on such factors. To test this hypothesis, thrombin formation was determined in PPP in the presence of LUVs containing either PC or PC/PS (1 : 1 molar ratio) to mimic quiescent or activated platelets, respectively. Thrombin formation was observed only in PPP with PC/PS LUVs and was not significantly affected by quercetin or catechin (Fig. 5C). These data indicate that the prothrombinase activity is strictly dependent on PS and suggest that, in PRP experiments, the effects of flavonoids resulted from inhibition of PS exposure on platelets. Addition of 100 nM annexin V, a protein that binds specifically to PS, completely abolished thrombin formation mediated by PC/PS LUVs in PPP (Fig. 5C), as well as in PRP activated with collagen or calcium ionophore (data not shown).

PPI metabolism in flavonoid-treated platelets

We first determined the effect of a pretreatment with different concentrations of quercetin and catechin on PIP and PIP_2 metabolism in resting platelets. Platelets were preincubated with or without the flavonoid for 20 min and then labeled with ^{32}P in the continuous presence of the drug. After extraction, PPI were separated by TLC. As shown in Fig. 6A, 100 μM quercetin reduced ^{32}P incorporation into PIP and PIP_2 by 25% and 60%, respectively. The inhibitory effect of quercetin on PIP_2 was significant from 5 μM and on PIP at 100 μM. The effects of catechin were weaker, inhibiting by 30% ^{32}P incorporation into PIP and PIP_2 at 100 μM (Fig. 6B). After stimulation with platelet agonists, PIP_2 is rapidly hydrolyzed by phospholipase C (PLC) and immediately resynthesized above its resting level [32,33]. To characterize the inhibitory effect of quercetin on PIP_2 resynthesis under the conditions used to study their effect on platelet functions, ^{32}P-labeled platelets were treated with or without quercetin for 30 min before stimulation for 10 min with collagen, calcium ionophore or thrombin (Fig. 6C). In the absence of the flavonoid, ^{32}P-labeled PIP_2 increased to 125–140% of the resting level, reflecting its resynthesis. An even greater increase was observed with PIP. When platelets were treated with quercetin before stimulation, PIP_2 and PIP resyntheses were reduced by about 50–70%. Inhibition of ^{32}P incorporation into PIP_2 by quercetin (100 μM) was the same, whether platelets were incubated with the drug for 40 min (Fig. 6C) or for 110 min (Fig. 6A).

Discussion

Flavonoids are multifunctional bioactive compounds, present in many plants, whose consumption has been linked with prevention of cardiovascular disorders. The present results demonstrate that quercetin and catechin inhibit platelet aggregation and secretion *in vitro*, as well as platelet procoagulant activity reflected by the transformation of prothrombin to thrombin. This latter effect is caused by inhibition of PS exposure, which has to be included in the spectrum of flavonoid cellular effects. Because thrombin acts as an important platelet agonist and is involved in the formation of atherosclerotic plaques [34], a possible decrease in the level of thrombin in blood plasma, caused by flavonoid consumption, can in part explain their protective effects against cardiovascular illness. Nevertheless, the mechanisms underlying the inhibitory effect of flavonoids on procoagulant platelet activity, such as PS exposure, are poorly understood.

PPI turnover is a key signal mechanism in platelets. In the first seconds after platelet activation, a specific pool of PIP_2 is hydrolyzed by PLC, followed by or coincident with a stimulation of PI-4 and PI(4)P-5 kinases, leading to increase the level of PIP_2 by 40% over its resting level [32]. The role of the PLC-mediated breakdown products of PIP_2, inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol, in promoting intracellular calcium increase and activation of PKC, respectively, has been extensively studied, but little is known about the role of the increase in the level of PIP_2 during platelet activation. For instance, the decrease in platelet levels of ^{32}P-PIP and ^{32}P-PIP_2 caused by oleic acid is associated with inhibition of platelet aggregation induced by platelet activating factor (PAF) [35]. platelet-derived growth factor (PDGF) at a concentration that completely inhibits collagen-induced platelet aggregation, prevents the replenishment of ^{32}P-PIP_2 after collagen-induced breakdown of ^{32}P-PIP_2 [36]. In our experiments, after ^{32}P labeling of PPI, quercetin and catechin were found to decrease the ^{32}P-PIP_2 level in resting platelets and to inhibit its resynthesis in platelets activated by different agonists.

The rapid externalization of PS during platelet activation is thought to be mediated by Ca^{2+} [15,17]. Quercetin, as well as catechin, prevents PS exposure and other features of platelet activation. The increase in $[Ca^{2+}]_i$ induced by collagen was inhibited by flavonoids, suggesting that the decrease in PS exposure could be partly explained by a reduced Ca^{2+} response due to the decreased level of PIP_2 and subsequent reduction of IP_3 production. In platelets activated with A23187, the decrease in PS exposure induced by flavonoids was not associated with inhibition of $[Ca^{2+}]_i$ increase. Under these conditions, the defect in PS exposure, associated with the diminution of PIP_2 concentration, suggests that PIP_2 could act as a Ca^{2+} target responsible for the induction of scrambling, as proposed in our previous studies [18–20].

We have previously reported that PIP_2 plays a role in phospholipid scrambling (including PS exposure) induced by Ca^{2+} or other polycationic effectors in erythrocytes [18,20] and in platelets [22]. A possible mechanism of action can involve the binding of polycationic factor(s), including Ca^{2+}, to PIP_2 located in the plasma membrane, destabilizing bilayer packing to the extent that loss of membrane asymmetry occurs. On the other hand, PIP_2 can induce phospholipid scrambling in red cells as a result of a redistribution of calcium ions [21], or can act as a putative cofactor of a scramblase [19], a calcium-dependent enzyme responsible for phospholipid scrambling [15,17].

Recent data underline the importance of the antioxidant activity of flavonoids on platelet function [13,37]. Flavonoids

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significantly inhibit the release of platelet H_2O_2 [13], whose production is associated with collagen-induced platelet aggregation. Whereas some reports argue in favor of a correlation between H_2O_2 production and PPI metabolism [38], or for a role of oxidation in phospholipid organization in apoptosis of various cells [39,40], no information is available on the effect of oxidants on PPI-kinase and PPI signaling pathways in platelets. We found that PS exposure induced by thrombin was not affected by tocoferol or catalase. This observation suggests that the antioxidant properties of flavonoids are unrelated to their inhibitory effect on platelet PS exposure. In summary, our data argue in favor of a role of PIP_2 in the antiplatelet effects of flavonoids, providing a potential mechanism for therapeutic targets.

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