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Apigenin Inhibits Platelet Adhesion and Thrombus Formation and Synergizes with Aspirin in the Suppression of the Arachidonic Acid Pathway

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Previous studies using washed platelets demonstrated that certain flavonoids inhibit platelet function through several mechanisms including blockade of TxA_2 receptors (TPs). We aimed to analyze the binding capacity of flavonoids to TPs in platelet rich plasma (PRP), investigated their effect in flowing blood, and evaluated the ability of apigenin to improve the efficacy of aspirin in the inhibition of platelet aggregation. The binding of flavonoids to TPs in PRP was explored using binding assays and the TP antagonist [^{3}H]SQ29548. Effects of flavonoids on platelet adhesion were assessed using arterial subendothelium with annular plate perfusion chambers, and global evaluation of apigenin on high-shear-dependent platelet function was determined by the PFA-100. To evaluate the ability of apigenin to potentiate the effect of aspirin, arachidonic acid-induced platelet aggregation was measured prior to and after consumption of subaggregatory doses of aspirin in the presence or absence of apigenin. Binding assays revealed that apigenin was an efficient competitor of [^{3}H]SQ29548 binding to PRP (K_i = 155.3 ± 65.4 μM), and perfusion studies showed that apigenin, genistein, and catechin significantly diminished thrombus formation when compared to control (26.2 ± 3.8, 33.1 ± 5.2, and 26.2 ± 5.2 vs 76.6 ± 2.6%, respectively; $p < 0.05$). Apigenin, similarly to the TP antagonist SQ29548, significantly prolonged collagen epinephrine-induced PFA-100 closure time in comparison to the control and, when added to platelets that had been exposed in vivo to aspirin, potentiated its inhibitory effect on platelet aggregation. The inhibitory effect of some flavonoids in the presence of plasma, particularly apigenin, might in part rely on TxA_2 receptor antagonism. There is a clear increase in the ex vivo antiplatelet effect of aspirin in the presence of apigenin, which encourages the idea of the combined use of aspirin and certain flavonoids in patients in which aspirin fails to properly suppress the TxA_2 pathway.

KEYWORDS: Flavonoid; apigenin; platelets; adhesion; aspirin

INTRODUCTION

Platelet activation is a critical step in the pathogenesis of thrombosis, which can lead to fatal and nonfatal myocardial or cerebral infarction and atherosclerosis (*1*). Prevention of those diseases relies on the use of antiplatelet agents (*2*); however, the consumption of phytochemicals, particularly flavonoids, has been shown to be beneficial for cardiovascular health as well (*3–5*). Although an explanation for the relationship between a diet high in fruit and vegetables and a decreased risk of thrombosis is far from clear, a number of studies have focused on the effects of dietary flavonoids and platelet function. In this context, flavonoids have been shown to impair enzymes involved in cellular signaling (*6–10*), to have anticoagulant activity (*11, 12*), and to enhance nitric oxide (NO) production (*13*).

Among the physiological agonists that activate platelets, arachidonic acid metabolites are of particular relevance. Thus, the ability of aspirin to inhibit thromboxane A_2 (TxA_2) synthesis, a potent inducer of platelet aggregation and a vasoconstrictor, is the primary rationale for its widespread use in recurrent myocardial infarction and thromboembolic disease (*14*). We have reported recently that certain flavonoids, particularly the flavone apigenin, found in parsley and celery, and the soy isoflavone genistein, compete for binding to the platelet TxA_2 receptor (TP) and subsequently abrogate downstream signaling,

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a modulatory effect that may be reproducible in myometrium smooth muscle and TP-transfected HEK 293T cells (*15, 16*).

The biological activity of flavonoids on platelets is often assessed by analyzing the activation of washed platelets in suspension. However, inherent flaws in such an approach are that polyphenols are not exposed to plasma, which may at a great extent influence their bioavailability. Also, the mechanism through which platelets are activated and form firm aggregates differs greatly between platelets in suspension and platelets adhering to an adhesive surface. For those reasons, in this study, we evaluated the binding capacity of certain flavonoids, particularly apigenin, to TPs in platelet rich plasma (PRP) and investigated the effect of these compounds in flowing blood using two experimental approaches: by the use of a perfusion method simulating physiologic flow conditions at moderate shear rates and by the use of the PFA-100 device to provide a global measure of high shear-dependent platelet function (*17, 18*). Finally, we evaluated in vitro the ability of apigenin to improve the efficacy of aspirin in the inhibition of platelet aggregation. This study provides new data supporting the potential use of flavonoids as an alterative or complementary antiplatelet therapy, which is nowadays a milestone in the prevention and treatment of cardiovascular disease.

MATERIAL AND METHODS

Materials. Apigenin, quercetin, catechin, rutin, and rhoifolin were kindly provided by Furfural Español S.A.-Nutrafur (Murcia, Spain). Genistein was from Sigma-Aldrich Química (Madrid, Spain). Flavonoids were solubilized in dimethylsulfoxide (DMSO) and stored frozen until use. In all experiments, the final concentration of DMSO was less than 0.4%. The TP antagonist SQ29548 {[1*S*-[1a,2a(*Z*),3a,4a]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2,2,1]hept-2-yl]-5-heptenoic acid} was purchased from Cayman Chemical (Ann Arbor, MI), and its tritium-labeled form ([^{3}H]SQ29548, specific activity 48.2 Ci/mmol^{-1}) was from PerkinElmer (Boston, MA). Arachidonic acid for platelet aggregation was obtained from Sigma-Aldrich (Madrid, Spain).

Blood Material. To participate in this study, informed consent was obtained from volunteer blood donors, according to the ethical standards of the committee of our institution. Whole blood was drawn into 4.5 mL vacuum-siliconated tubes containing 0.5 mL of 0.129 M sodium citrate. For perfusion studies, blood was collected into a citrate−phosphate−dextrose (CPD) solution (100 mM sodium citrate, 16 mM citric acid, 18 mM sodium hydrogen phosphate, and 130 mM dextrose) (at a final citrate concentration of 19 mM).

[^{3}H]SQ29548 Binding Assays. PRP was prepared by centrifugation of citrated whole blood at 150*g* for 10 min, and the top two-thirds was transferred by careful pipetting into a separate tube. Platelet poor plasma (PPP) was prepared by a further centrifugation step of the same tube at 1000*g* for 20 min and used to adjust PRP to a final count of 200×10^9 L^{-1}. Cold saturation binding assays with the TP antagonist SQ29548 were performed by incubating PRP at room temperature with 5 nM [^{3}H]SQ29548 in the presence of increasing concentrations of unlabeled SQ29548 (0–500 nM) in a final volume of 250 μL of SQ buffer (10 mM Tris-HCl, 120 mM NaCl, 5 mM D-dextrose, and 0.8 μM indomethacin, pH 7.4) for 30 min. Nonspecific binding was measured in the presence of 10 μM SQ29548. To assess the interaction of flavonoids with TPs, competition binding assays were carried out essentially as above but incubating PRP with [^{3}H]SQ29548 and increasing concentrations of apigenin (0–4 mM) or with other flavonoids (250 μM). In either case, platelet-bound ligand was separated by filtration through glass fiber filters (Millipore, Bedford, MA) under vacuum. Filters were subsequently rinsed with ice-cold SQ buffer and transferred to vials containing 5 mL of scintillation fluid. Radioactivity was measured in a liquid scintillation counter (Wallac, Turku, Finland) for 1 min. Data from binding experiments were analyzed with the LIGAND software package (Biosoft, Cambridge, United Kingdom) using the COLD or DRUG option as appropriate. This computer analysis determined the SQ29548 binding sites per platelet (B_{max}), ligand affinity (K_d), and the apigenin inhibition constant (K_i).

A set of binding assays were performed to assess the effect of human serum albumin (HSA) on SQ29548 and apigenin binding to washed platelets. In these experiments, washed platelets in 2 mM PBS-EDTA (200×10^9 platelets/L^{-1}) (*16*) were incubated as above with 5 nM [^{3}H]SQ29548, in the absence or presence of 4% HSA and/or 200 μM apigenin. Platelet-bound ligand was separated and quantified as above.

Platelet Adhesion and Fibrin Formation on Subendothelial Surfaces Exposed to Flavonoid-Treated Blood under Flow Conditions. Enzymatically denuded New Zealand rabbit aorta segments were perfused with CPD anticoagulated blood at 37 °C in annular chambers according to Baumgartner (*19*). Blood samples ($n = 5$) nonincubated or incubated with flavonoids (200 μM) were recirculated through the chamber for 10 min at a shear rate of 800 s^{-1} using a peristaltic pump (Renal Systems, Minneapolis, MN). At the end of the perfusion, the rods were removed from the chamber, and the segments were rinsed with 20 mL of phosphate-buffered saline (PBS), sliced off, and washed with Milloning's buffer (140 mM sodium phosphate with 300 mM glucose, pH 7.2), containing 4% formaldehyde and 1% glutaraldehyde. The fixed segments were histologically processed as previously described (*20*).

Platelet interaction with subendothelium was evaluated as described elsewhere (*21*) and classified as follows: contact (C), platelets that were attached but not spread on the subendothelium; adhesion (A), platelets that were spread on subendothelium and formed layers of less than 5 μm in height; and thrombi (T), platelet aggregates of 5 μm or more in height. The total covered surface (CS) was obtained by adding C + A + T and was expressed as the percentage of total surface of the vessel screened. %T refers to the percentage of CS with thrombi with more than 5 μm in height.

Platelet Function Analysis of Flavonoid-Treated Whole Blood Using the PFA-100 System. Citrated whole blood samples were obtained from volunteers ($n = 7$) with normal platelet counts and hematocrits and analyzed within 4 h in duplicate on the PFA-100 system (Dade Behring International, Miami, FL). The PFA-100 measures platelet function by exposing platelets to micropore membranes containing either collagen/epinephrine (CEPI) or collagen/ADP (CADP), at high shear flow rates (5000–6000 s^{-1}) (*17*). Samples were incubated undisturbed for 30 min at 25 °C with 50–200 μM flavonoids (apigenin or rutin, as representative of polyphenols competing or not for TPs, respectively), with 1 μM SQ29548 or 0.4% DMSO as control, prior to analysis. The reference ranges were 85–165 s for CEPI and 71–118 s for CADP.

Platelet Aggregation Studies. PRP (300×10^9 platelets/L^{-1}) from four volunteers was incubated with increasing concentrations of apigenin or rutin at room temperature for 30 min and was stimulated with collagen (1 μg/mL) or arachidonic acid (750 μM), which are two agonists dependent on the TxA2 pathway (*15*). The aggregation response was followed by an aggregometer (Aggrecorder II, Menarini Diagnostics, Florence, Italy) as the percentage of light transmission, with PRP as the baseline and PPP as 100%. Aggregation was allowed to proceed for 300 s, and curves were quantified as the area under the curve using the MIP4 advanced system software (Microm España, Barcelona, Spain).

To analyze synergism between apigenin and aspirin, three healthy adults that had not taken nonsteroidal antiinflammatory medication for more than 7 days were included. Venous blood was obtained from each subject following an overnight fast on two separate test days: prior to any treatment and 3 h after consumption of a single dose of 0.25 mg/kg aspirin. Citrated PRP (300×10^9 platelets/L^{-1}) was incubated with DMSO and 200 μM apigenin or rutin, as above, prior to stimulation with 750 μM arachidonic acid. Time−course changes in light transmission—as the percentage of light transmission—were recorded.

Statistical Analysis. Unless stated specifically, data are expressed as means ± standard deviations (SD) from at least three experiments performed in different samples. Statistical comparisons were achieved by two-tailed Student's t test using GraphPad Prism for Windows

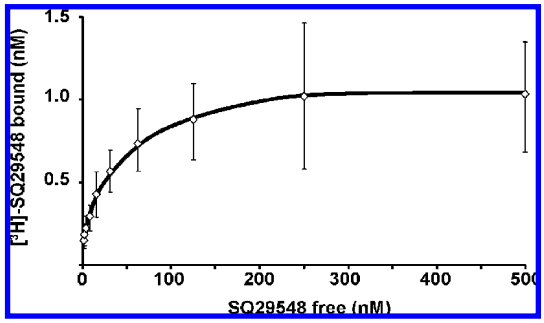

Figure 1. Saturation curve of [^{3}H]SQ29548-specific binding to PRP. PRP was incubated at room temperature with 5 nM [^{3}H]SQ29548 in the presence of increasing concentrations of unlabeled SQ29548 (0–500 nM). Using the COLD option of the computer program LIGAND, the displacement curves obtained were transformed into saturating plots. The plot represents means ± SD from three experiments.

version 4.0 (GraphPad Inc., San Diego, CA). Differences were considered to be significant at $p < 0.05$.

RESULTS

Effect of Flavonoids on [^{3}H]SQ29548 Binding to PRP. According to our previous data, certain flavonoids (apigenin at a great extent) behave as selective TP antagonists in a system free of plasma proteins (*15, 16*). Herein, we analyzed the interaction of apigenin with TPs in the presence of plasma, by means of binding assays using [^{3}H]SQ29548 as a TP tracer. Under these experimental conditions, LIGAND analysis demonstrated that SQ29548 equilibrium binding data best fitted to a single class of sites, with an apparent K_d of 30.9 ± 10.4 nM and a density (B_{max}) of 4348 ± 590 sites/platelet (**Figure 1**). As illustrated in **Figure 2A**, apigenin was confirmed to be an efficient inhibitor of [^{3}H]SQ29548 binding to PRP in a dose-dependent way (K_i = 155.3 ± 65.5 μM). Scatchard analysis of cold saturation assays of [^{3}H]SQ29548 performed in the absence or presence of 250 μM apigenin (n = 3) demonstrated a significant reduction in the number of [^{3}H]SQ29548 binding sites (B_{max}, 3583 ± 1837 vs 1987 ± 1325; P = 0.0377), with negligible effect on the prostanoid affinity to PRP (K_d, 27.89 ± 0.86 vs 33.7 ± 21.1 nM; P = 0.6971). These results are consistent with a noncompetitive inhibition of apigenin to TP in PRP. As shown in **Figure 2B**, not only apigenin but also genistein (250 μM) effectively competed for TxA_2 binding sites, in good agreement with their effect on washed systems (*15, 16*). Noteworthy, catechin, in concordance with results from perfusion studies described below, exhibited a moderate competing effect, while quercetin, a compound that had previously been shown to compete with moderate affinity for TxA_2 binding sites in the absence of plasma (*15, 16*), displayed negligible competition for TP in PRP. Glycosylated flavonoids displayed different behaviors. Thus, rhoifolin displayed significantly reduced capacity to bind to TPs as compared to its nonglycosylated counterpart (apigenin), indicating that the 7-*O*-glycosylation exerts a negative steric influence on the TP affinity. By contrast, rutin shows a similar binding capacity for TPs than the corresponding aglycon (quercetin), suggesting that the 3-*O*-glycosylation that increases the electronic stability of the C_2=C_3 double bond of flavonoid skeleton has little effect in the interaction with TPs. Finally, we found that HSA inhibited by 55% the [^{3}H]SQ29548 binding to washed platelets and decreased more than 3-fold the competition of apigenin for such binding to TPs (data not shown).

Effects of Flavonoids on Platelet Interaction with the Subendothelium. To assess the role of flavonoids in the interaction of platelets with subendothelium, we carried out perfusion assays with vessel segments at moderate shear rates, and morphometric evaluation was performed using a computerized system. Perfusion of citrated whole blood in the absence of flavonoids resulted in the formation of platelet thrombi. Quantification of the data showed that the %CS in control samples after 10 min of perfusion was of 25%. In the apigenin- and genistein-treated samples, %CS was significantly reduced (18 and 21%, respectively) (**Figure 3A**). Aggregates of more than 5 μm in height (%T) were found covering 77% of the CS in the absence of flavonoids, while blood samples that had been preincubated with apigenin or genistein showed a significant decrease in the percentage of the largest thrombi observed (26 and 33%, respectively). Catechin did not modify the total CS but significantly decreased the surface covered with aggregates of more than 5 μm in height (26%) with respect to control (P < 0.05) (**Figure 3B**). When the perfusion was carried out in the presence of rutin, rhoifolin, or quercetin, morphometric parameters remained at control levels.

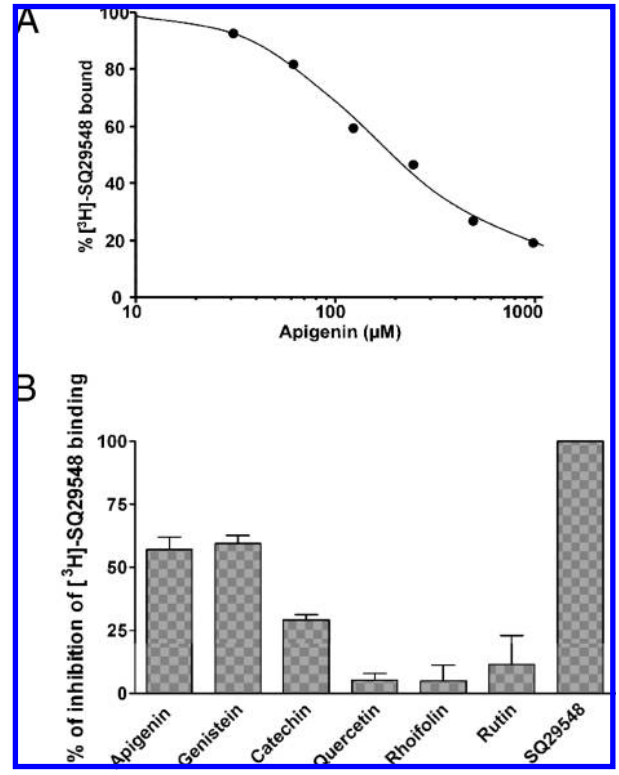

Figure 2. Effect of flavonoids on [^{3}H]SQ29548 binding to PRP. PRP was incubated with [^{3}H]SQ29548 (5 nM) in the presence of increasing concentrations of apigenin (**A**) or a fixed concentration of different flavonoids (250 μM) as competitors (**B**). The plot shown in panel **A** is a representative example of the resultant dose-dependent displacement curves of the specific binding by apigenin (n = 3). Panel **B** represents the percentage of [^{3}H]SQ29548-specific inhibition, considering that 100% of inhibition was achieved in the presence of unlabeled SQ29548. Results are means ± SD from three experiments.

Effects of Flavonoids on Platelet Function Monitored with PFA-100. To approach the idea of flavonoids negatively affecting platelet function, we analyzed the antagonist efficacy of apigenin and rutin, the former but not the latter having shown to abrogate downstream TP signaling (*16*), in the reduction of blood flow ensuring plug formation with PFA-100 cartridges. To monitor platelet hypofunctionality induced by flavonoids, we used the most sensitive CEPI cartridges and also CADP cartridges.

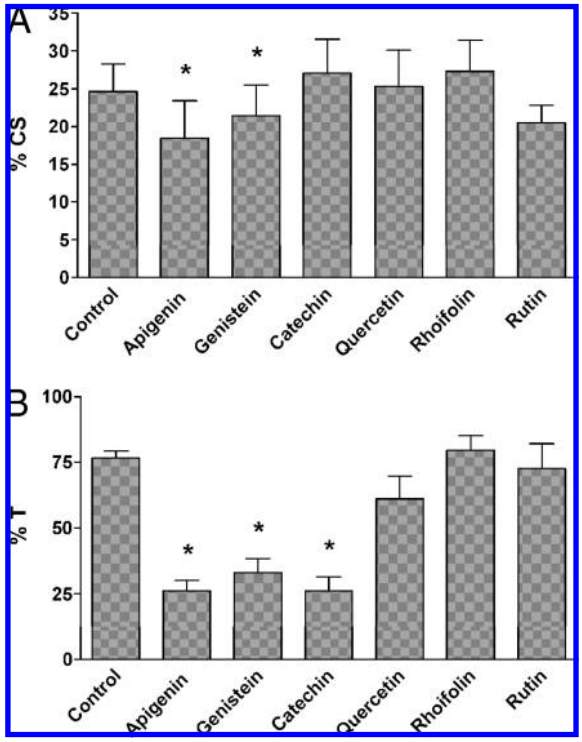

Figure 3. Bar diagram expressing percentages of the vessel surface covered by platelets in perfusion experiments performed in the absence or presence of different flavonoids (200 μM). Panel **A** indicates the surface covered by platelets, while panel **B** shows the surface covered by groups of platelets forming aggregates >5 μm in height (thrombi). *$P < 0.05$ with respect to the control group. Results are means ± SD from five experiments.

Apigenin at 200 μM significantly prolonged CEPI-induced closure times as compared to control (164 ± 73 vs 104 ± 15 s), while rutin exerted no effect (114 ± 19 s) (**Figure 4A**). There was a significant correlation between CEPI closure times ($r = 0.6939$ and $P = 0.0007$) and the concentration of apigenin in the samples (**Figure 4B**). Noteworthy, the TP antagonist SQ29548 (1 μM) prolonged the CEPI closure times (167 ± 65 s) to a similar extent than apigenin (**Figure 4A**). In contrast, PFA closure times using CADP did not evidence any delay in the presence of either flavonoid or SQ29548 ($P > 0.05$; data not shown), consistent with the inability of apigenin at this concentration to inhibit ADP-induced platelet aggregation (data not shown).

Platelet Aggregation. Because we aimed to investigate the effect of flavonoids on the platelet TxA_2 pathway, we assessed, under our experimental conditions, platelet aggregation induced by agonists that were found to be dependent on the TxA_2 pathway (collagen and arachidonic acid). We observed that short incubation of platelets with apigenin caused a long lag phase before secondary aggregation, resulting in significant reduced values of area under the curve at 400 and 800 μM, both on arachidonic acid- and collagen-induced responses (**Figure 5A**). By contrast, rutin had a negligible effect (data not shown).

To investigate whether or not synergism exists between aspirin and apigenin, we studied their combined effect on platelet aggregation. Neither aspirin consumption at 0.25 mg/kg (16.8 ± 3.3 mg) nor apigenin or rutin at a concentration of 200 μM added in vitro to PRP inhibited arachidonic acid-induced platelet aggregation (**Figure 5B**). Noteworthy, platelets that had been exposed in vivo to subaggregatory concentrations of aspirin when incubated with apigenin displayed a fully abrogated response to arachidonic acid-induced aggregation ($p < 0.05$).

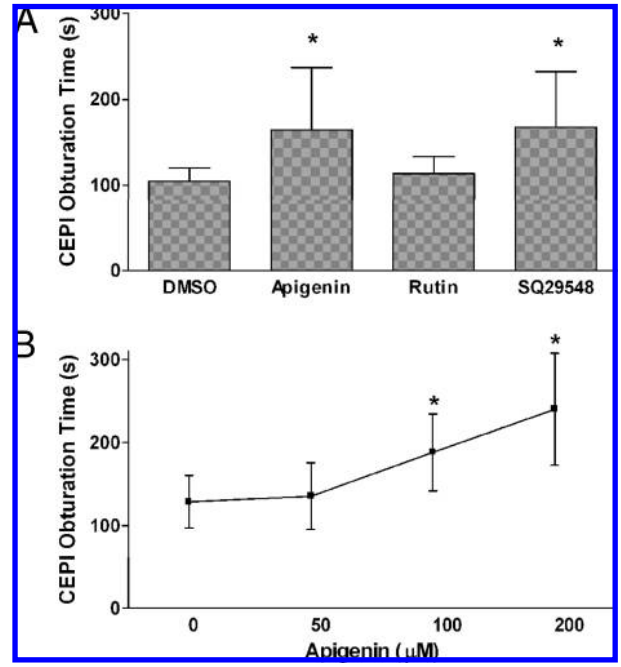

Figure 4. Effects of apigenin on collagen-epinephrine (CEPI)-induced closure time. Panel **A** shows CEPI-induced closure time in the presence of SQ29548 (1 μM), apigenin (200 μM), or rutin (200 μM). Panel **B** shows the apigenin concentration-dependent increased PFA-100 CEPI-induced closure times. *$P < 0.05$ vs corresponding control values. Results are means ± SD from seven experiments.

This trend was not observed for rutin, as platelets that had been exposed to aspirin did not significantly modify the aggregation response as compared to aspirin alone or rutin alone-treated platelets ($P > 0.05$) (**Figure 5B**).

DISCUSSION

The present study supports the concept that flavonoids can modify platelet function in plasma rich systems. In this sense, platelet adhesion measured by perfusion studies was lower after incubation of blood with apigenin and genistein. Also, apigenin caused an SQ29548-like effect on platelet function, as measured in terms of platelet-related primary hemostasis evaluated with PFA-100. Moreover, apigenin potentiated the antiplatelet effect of aspirin in PRP. Our previous data indicate that apigenin exhibits a negligible inhibitory effect neither upon ADP activation (unpublished observations) nor on thrombin-induced responses in aspirinized platelets (*15, 16*) and strongly suggest that the inhibitory effect of certain flavonoids is related to their ability to antagonize TPs. The current findings further indicate that, in good agreement with their effect on washed systems, apigenin and genistein interact with TPs in PRP, suggesting a direct relationship between inhibition of platelet function and antagonism of TPs.

Dietary flavonoids have received much attention due to their biological activity. It is clear that these components must, by definition, be bioavailable in some form to exert biological effects. The biological activity of polyphenols is often assessed by using cultured cells or washed cell systems as experimental models to report a concentration of these substances that elicit a response. However, phenolic compounds undergo modifications after being metabolized and can also interact with proteins and enzymes present in the blood, which may significantly modify the bioactivity of such compounds (*22, 23*). Thus, flavonoids display moderate affinities for albumin (*24, 25*)

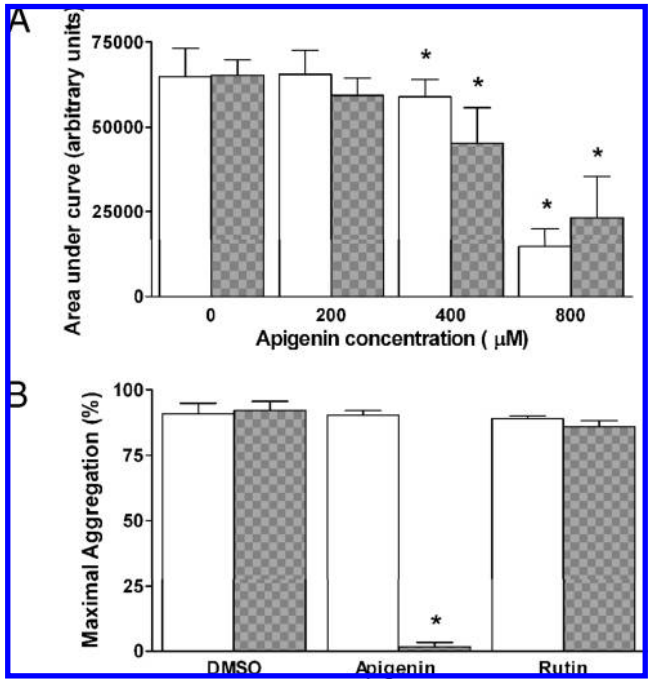

Figure 5. Effect of apigenin on platelet aggregation. Panel **A** shows the area under the curve after stimulation with 750 μM arachidonic acid (white bars) or 1 μg/mL collagen (gray bars) in the presence of increasing concentrations of apigenin. *$P < 0.05$ vs platelets incubated with DMSO. Results are means ± SD from four experiments. Panel **B** illustrates the effect of apigenin or rutin (200 μM) on arachidonic acid (750 μM)-induced platelet aggregation prior to (white bars) or 3 h after consumption of 0.25 mg/kg aspirin (gray bars). *Significantly different from platelets exposed to aspirin alone and from nontreated platelets incubated with the specific flavonoid. Results are means ± SD from three experiments.

and other proteins, which may result in changes in their physicochemical and structural properties, potentially influencing the binding of these compounds to certain receptors to exert their activity. In the context of the present study, attention was given to the analysis of the biological activity of flavonoids on platelets in the presence of plasma proteins. In PRP, apigenin and genistein effectively competed for TxA_2 binding sites, in good agreement with its effect in washed system (*15*). Yet, the affinity of this flavonoid for the TxA_2 binding sites in the presence of plasma proteins was more than 30-fold lower than in washed platelets (*15*). These data suggest that flavonoids have relatively high plasma proteins binding ability, which might interfere with their interaction with TPs. This is also supported by our observation that HSA significantly reduced the binding of SQ23548 and apigenin to TPs in washed platelets. For catechin, a lower decrease in its affinity with TPs in PRP was observed as compared to a washed system (*15*), suggesting that this flavonoid might have a lower capacity to interact with plasma proteins.

The comparison of the different capacities of the evaluated flavonoids to compete for TxA_2 binding sites in the PRP system further confirms and extent our previous findings (*15*) that, regarding the flavonoid−TP interaction, the active structure of flavonoid skeleton involves the combination of carbons 7 and 8 of the A ring, the lactone group conjugated with the C_2═C_3 double bond on the C ring, and the B ring carbons 2′, 3′, and 4′. The flavonoid affinity for TP also depends on the presence of hydroxyl radicals on carbons 7 and 4′ (apigenin and genistein). The presence of more complex radicals within the mentioned "active" flavonoid skeleton, as glycosides in rutin and rhoifolin, significantly impairs the affinity for TP, most likely by modification of the steric and electrostatic molecular environment.

Tethering of platelets to a damaged vessel wall via GPIb-IX-V interacting with collagen-bound VWF is the first step in hemostasis (*26*), and further activation and stable attachment are mediated through GPVI/Src kinases and integrin $\alpha 2\beta 1$ and subsequent binding of fibrinogen to platelet GPIIb/IIIa (*27*). Data from our perfusion studies showed that apigenin and genistein induced a significant reduction in the overall ability of platelets to interact with the endothelium (CS). Moreover, these compounds, and also catechin, significantly associated with a more pronounced decrease in the morphometric parameters that quantify the building of large aggregates onto previously spread platelets, consistent with the idea that certain flavonoids could interfere with mechanisms mediating platelet−platelet interactions. The ability of apigenin and genistein to interfere with both the adhesive and cohesive functions of platelets resembles that described in previous studies with a TxA_2 antagonist (*28*). This effect differs from that observed with aspirin since it decreases the aggregate formation although it is unable to block the adhesive function of platelets (*20*). This discrepant effect could be explained on the basis of their differential mechanism of action. While aspirin is known to inhibit TxA_2 synthesis, thromboxane antagonism would counteract the effects of any minimal amount of TxA_2. Therefore, TxA_2 antagonism, such as that exhibited by apigenin and genistein, could be potentially superior in terms of antiplatelet effect.

Further support for the effects of flavonoids, particularly apigenin, on platelet function came from the PFA-100 system using CEPI and CADP cartridges. The CEPI test is highly sensitive to detect qualitative platelet abnormalities induced by pharmacological agents such as aspirin and other nonsteroidal antiinflammatory agents (*29, 30*). Closure time by the CEPI system, but not by the CADP, was prolonged after the addition of apigenin, suggesting aspirin-like effects on hemostasis. The observation that SQ29548 prolonged the CEPI closure time correspondingly further supports the hypothesis that antagonism of TP might, to a great extent, mediate the effect of apigenin. These findings are in good agreement with recent data showing that consumption of flavonoid-rich foods, such as red wine, cocoa, or chocolate, can also prolong PFA closures times (*31, 32*). Additionally, data from aggregation studies evidenced that apigenin exhibited a dose-dependent inhibition of arachidonic acid- and collagen-induced aggregation, a response highly dependent on the TxA_2 pathway. Together, these data suggest that TP antagonism is a key event in the observed biological effects. However, additional mechanisms may be involved and cannot be ruled out. Thus, apigenin is also an inhibitor of tyrosine kinases (*16, 33*), an important pathway for collagen signaling leading to platelet aggregation.

During the last 30 years, aspirin has saved the lives of millions of patients with cardiovascular disease, due to its antithrombotic properties via inhibition of platelet TxA_2 synthesis. However, the variable effectiveness of aspirin in terms of clinical outcomes and laboratory findings is nowadays considered a real clinical problem (*34*). Previous observations support that the combination of antiplatelet drugs with preparations of herbal origin—food supplements (*32, 35, 36*) may be beneficial in some clinical states and that a synergism exists between flavonoids and aspirin on their inhibitory effect of platelet function (*32, 37*). This study confirms and extends such evidence. Thus, in our experimental conditions, neither apigenin nor the exposure of platelets to subthreshold concentrations of aspirin inhibited platelet aggregation induced by arachidonic acid when used alone but

essentially potentiated their inhibitory effect when used together, according to the direct effects of flavonoids on platelet inhibition on the cyclooxygenase−TxA_2 pathway (*15, 16, 38–40*). Although the effects of apigenin alone on platelet aggregation are negligible, they may be significant in compounding and/or correcting an incomplete therapeutic effect of aspirin. Because even the so-called apigenin rich foods contain limited amounts of this flavonoid and its absorption might hardly exceed 10%, it is unlikely that normal ingestion of these foods may lead to apigenin plasma levels that significantly affect the TP−TxA_2 pathway. However, it might be that pharmacological supplementation with purified apigenin, with an extremely safe profile, could provide high apigenin plasma levels that might allow the use of aspirin at lower concentrations to inhibit platelet aggregation. Additional research will be needed to establish the bioavailability of apigenin, identify its plasma active metabolite, and elucidate the usefulness of its association with aspirin for patients suffering from atherothrombotic processes.

In conclusion, using an in vitro experimental approach being closer to physiological conditions than washed isolated platelets, some flavonoids, particularly apigenin, have been found to modulate platelet reactivity. The inhibitory effect of these compounds might in part rely on TP antagonism and encourages the idea of the combined use of aspirin and certain flavonoids in patients in which aspirin fails to properly suppress the TxA_2 pathway, leading to aspirin resistance. Further in vivo studies are expected to analyze whether pharmacological supplementation of these compounds may correct an incomplete therapeutic effect of aspirin in the prevention or treatment of thrombotic events.

ABBREVIATIONS USED

A, adhesion platelets; CADP, collagen/ADP cartridge; CEPI, collagen/epinephrine cartridge; C, contact platelets; CS, covered surface by platelets; DMSO, dimethylsulfoxide; GP, glycoprotein; HSA, human serum albumin; NO, nitric oxide; PRP, platelet rich plasma; T, thrombi; TPs, thromboxane A_2 receptors; TxA_2, thromboxane A_2.

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