The flavonoids quercetin and catechin synergistically inhibit platelet function by antagonizing the intracellular production of hydrogen peroxide^1,2

Pasquale Pignatelli, Fabio M Pulcinelli, Andrea Celestini, Luisa Lenti, Andrea Ghiselli, Pier Paolo Gazzaniga, and Francesco Violi

ABSTRACT

Background: Epidemiologic studies have shown an inverse relation between moderate consumption of red wine and cardiovascular disease. Studies have shown that red wine and its component flavonoids inhibit in vivo platelet activation, but the underlying mechanism has not yet been identified.

Objective: Because we showed previously that collagen-induced platelet aggregation is associated with a burst of hydrogen peroxide, which in turn contributes to stimulating the phospholipase C pathway, the aim of this study was to investigate whether flavonoids synergize in inhibiting platelet function and interfere with platelet function by virtue of their antioxidant effect.

Design: We tested the effect of 2 flavonoids, quercetin and catechin, on collagen-induced platelet aggregation and hydrogen peroxide and on platelet adhesion to collagen.

Results: Catechin (50–100 μmol/L) and quercetin (10–20 μmol/L) inhibited collagen-induced platelet aggregation and platelet adhesion to collagen. The combination of 25 μmol catechin/L and 5 μmol quercetin/L, neither of which had any effect on platelet function when used alone, significantly inhibited collagen-induced platelet aggregation and platelet adhesion to collagen. Such a combination strongly inhibited collagen-induced hydrogen peroxide production, calcium mobilization, and 1,3,4-inositol triphosphate formation.

Conclusions: These data indicate that flavonoids inhibit platelet function by blunting hydrogen peroxide production and, in turn, phospholipase C activation and suggest that the synergism among flavonoids could contribute to an understanding of the relation between the moderate consumption of red wine and the decreased risk of cardiovascular disease. Am J Clin Nutr 2000;72:1150–5.

KEY WORDS Platelet aggregation, flavonoids, hydrogen peroxide, platelet adhesion, phospholipase C, quercetin, catechin, red wine, cardiovascular disease

INTRODUCTION

Moderate consumption of red wine is associated with a decrease in the incidence of cardiovascular events (1, 2). Components of red wine, such as flavonoids, have been implicated in such cardiovascular benefits because of their ability to inhibit platelet function. In fact, in vivo experimental studies in animals showed that both red wine and grape juice decreased platelet activation in stenosed canine coronary arteries (3). A similar effect was observed with the flavonoids quercetin and catechin, indicating that these components of red wine were involved in eliminating the flow reduction due to platelet activation (4). Several in vitro studies showed that flavonoids such as resveratrol, quercetin, and catechin inhibit platelet aggregation; nevertheless, a potential limitation of these studies was that the concentration used to achieve this inhibition was too high. Accordingly, some authors questioned the antiplatelet activity exerted in vivo by these red wine components (5). Note that investigations of the effects of flavonoids on platelet function to date focused on each component singly; whether flavonoids act synergistically to inhibit platelet activation has never been studied. Because more than one flavonoid circulates in human blood after red wine consumption, such synergism might be relevant because lower concentrations of flavonoids than those investigated previously could modulate platelet activity.

Another issue inherent to the antiplatelet effect of flavonoids is their mechanism of action. Even if the results of most studies show that flavonoids interact with arachidonic acid metabolism, thus inhibiting platelet thromboxane A_2 production, the underlying mechanism has never been investigated (6, 7). Flavonoids are phenolic compounds, the antioxidant effects of which are related to radical scavenging rather than to metal chelation (8). It has been suggested that the inhibition of both platelet function and arachidonic acid metabolism depends on antioxidant activity, but no studies have investigated whether flavonoids interact with oxidant species formed on platelet activation. The aim of this study, therefore, was to investigate whether flavonoids 1) act synergistically to inhibit platelet function and 2) interfere with platelet function by virtue of an antioxidant effect.

^1From the Department of Experimental Medicine and Pathology, Institute of 1st Clinical Medicine, University La Sapienza, National Institute for Nutrition, Rome.
^2Address reprint requests to PP Gazzaniga, Dipartimento di Medicina Sperimentale e Patologia, Università degli Studi di Roma La Sapienza, Viale Regina Elena 324, 00161 Rome, Italy. E-mail: gazzaniga@uniroma1.it.

Received November 2, 1999.
Accepted for publication April 5, 2000.
PLATELET INHIBITION BY CATECHIN AND QUERCETIN

MATERIALS AND METHODS

Materials

$^{32}$Pi and [3H]oleic acid were from Amersham (Arlington Heights, IL). Fura 2-AM and 2', 7'-dichlorofluorescein diacetate (DCFH-DA) were from Molecular Probes (Eugene, OR) and Sepharose 2B was from Pharmacia (Uppsala, Sweden). Tetrapeptide Arg-Gly-Asp-Ser (RGDS) was from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Type I collagen was from Mascia Brunelli (Milan, Italy). HPLC columns (Partisil 10 SAX) were from Whatman (Clifton, NJ). Bovine serum albumin, HEPES, acetylsalicylic acid, catechin, quercetin, fibrinogen, inorganic pyrophosphatase, digitonin, formaldehyde, indomethacin, creatine phosphate, and creatine phosphokinase were from Sigma Chemical Co (St Louis).

Platelet preparations

Human blood from drug-free, healthy volunteers was anticoagulated with citric acid:citrate:dextrose (9). Platelet-rich plasma was centrifuged at 800 × g for 20 min at room temperature and the pellet was suspended in a volume half that of the initial volume of autologous, platelet-poor plasma. The platelet suspensions were incubated for 1 h at 37°C with 3 μmol Fura 2-AM/L, 40 μmol DCFH-DA/L, 7.4 GBq (2 Ci) $^{32}$Pi/L, or 3.7 MBq (1 mCi) [3H]oleic acid/L. Platelets were washed by gel filtration on Sepharose 2B by using Ca2+-free Tyrode’s solution, which rapidly diffuses across cell membranes and is then trapped within the cell via a deacetylation reaction. In the presence of hydrogen peroxide, this compound is oxidized to dichlorofluorescein (DCF), which is highly fluorescent (17). The effect of scalar concentrations of quercetin and catechin on hydrogen peroxide production induced by 10 and 20 mg collagen/L are shown in Figure 1. Compared with untreated platelets, collagen-stimulated platelets enhanced the production of hydrogen peroxide, which was dependent on the concentration of collagen used. Catechin and quercetin inhibited collagen-induced hydrogen peroxide production by 10 and 20 mg collagen/L, respectively. Neither compound alone had any inhibitory effect (data not shown).

Flow cytometric analysis and platelet aggregation

DCFH-DA was added to GFPs (final concentration: 40 μmol/L); after 15 min incubation with or without catechin and quercetin. GFPs were activated with collagen. The reaction was stopped with 2 mmol EGTA/L after 1 min. Samples were analyzed on a Coulter XL-MCL (Hialeah, FL) flow cytometer equipped with an argon laser (480-nm emission) set up to measure logarithmic forward light scatter, which is a measure of particle size; logarithmic 90° light scatter, which is a measure of cell granularity; and green (DCF) 510–550-nm fluorescence. The fluorescent signal generated by the probe was expressed as the stimulation index, which is the mean channel fluorescence intensity of stimulated platelets/mean channel fluorescence intensity of unstimulated platelets. In vitro platelet aggregation was evaluated according to Born (10). Collagen was used at concentrations of 2–4 mg/L.

Platelet cytosolic Ca$^{2+}$ concentrations

Platelet cytosolic Ca$^{2+}$ concentrations were measured by using the fluorescent indicator dye Fura-2, according to Grynkiewicz et al (11); the changes in fluorescence were then monitored with an SFM 25 fluorimeter (Kontron, Zurich, Switzerland) set at 340-nm excitation and 510-nm emission wavelengths.

Phospholipase C activation and platelet adhesion to collagen

1,3,4-Inositol triphosphate (IP₃) production, an indicator of phospholipase C activation, was analyzed 30 s after platelet stimulation according to Pulcinelli et al (12). Collagen was used at a concentration of 10 mg/L, which was the lowest concentration able to induce a reproducible response. [3H]Oleic acid–labeled platelet suspensions were used to evaluate platelet adhesion to collagen (50 mg/L) according to Smith and Dangelmaier (13).

Statistical analysis

Data are reported as means ± SEMs. The responses under different experimental conditions were compared by using Student’s t test and the Bonferroni test to assess specific differences between groups. Significance was set at P < 0.05. STATVIEW (Abacus Concepts Inc, Berkeley, CA) was used for the analysis.

RESULTS

Flow cytometric analysis

Flow cytometry uses the properties of DCFH-DA (14–16), which rapidly diffuses across cell membranes and is then trapped within the cell via a deacetylation reaction. In the presence of hydrogen peroxide, this compound is oxidized to dichlorofluorescein (DCF), which is highly fluorescent (17). The effect of scalar concentrations of quercetin and catechin on hydrogen peroxide production induced by 10 and 20 mg collagen/L are shown in Figure 1. Compared with untreated platelets, collagen-stimulated platelets enhanced the production of hydrogen peroxide, which was dependent on the concentration of collagen used. Catechin and quercetin inhibited collagen-induced hydrogen peroxide production by 10 and 20 mg collagen/L, respectively. Neither compound alone had any inhibitory effect (data not shown).

Platelet aggregation

The effect of catechin and quercetin on platelet aggregation was measured by using 2 different concentrations of collagen. Both catechin and quercetin inhibited collagen-induced platelet aggregation. The degree of inhibition was dependent on the concentration of collagen used. Thus, 100 μmol catechin/L inhibited ~75% platelet aggregation induced by 2 mg collagen/L, and inhibited ~39% platelet aggregation induced by 4 mg collagen/L. In platelets treated with 20 μmol quercetin/L, the degree of inhibition of collagen-induced platelet aggregation (2 and 4 mg/L) was 50% and 43%, respectively. The combination of 25 μmol catechin/L and 5 μmol quercetin/L, which did not affect platelet aggregation when used alone, significantly inhibited (55%) platelet aggregation induced by both concentrations of collagen (Figure 2).

Changes in intracellular calcium concentration

Calcium mobilization, expressed as a percentage change in intracellular calcium concentration, was inhibited by catechin and quercetin. In platelets stimulated with 4 mg collagen/L, 100 μmol catechin/L, and 20 μmol quercetin/L significantly decreased calcium mobilization by 71% and 65%, respectively.
The incubation of platelets with 25 \( \mu \text{mol} \) catechin/L plus 5 \( \mu \text{mol} \) quercetin/L significantly inhibited calcium mobilization by 71%. A similar result was observed when calcium mobilization was induced by 8 mg collagen/L (Figure 3).

**Phospholipase C activation**

\[^{32}\text{P}]\text{IP}_3\) production in collagen-stimulated platelets was inhibited by catechin and quercetin: 10 mg collagen/L, 100 \( \mu \text{mol} \) catechin/L, and 20 \( \mu \text{mol} \) quercetin/L significantly decreased \( \text{IP}_3\) production by 50% and 93%, respectively. The incubation of platelets with 25 \( \mu \text{mol} \) catechin/L plus 5 \( \mu \text{mol} \) quercetin/L significantly inhibited \( \text{IP}_3\) production by 72%; similar effects were observed when platelets were stimulated with 20 mg collagen/L (Figure 4), but the degree of inhibition was less, although still significant.

**Platelet adhesion to collagen**

Activation of platelets by collagen is a multistep event. In fact, after an initial attachment to platelets through second messenger pathways, collagen stimulates release of thromboxane and ADP, which are important platelet agonists that induce aggregation (18). To study platelet adhesion to collagen (50 mg/L) without the interference of aggregation and activation induced by all the known agonists released from platelet granules on collagen stimulation, platelets were preincubated with the cyclooxygenase inhibitor aspirin, with the ADP-removing system creatine phosphate and creatine phosphokinase, and with the fibrinogen-fibronectin antagonist RGDS (13).

The adhesion of platelets to 50 \( \mu \text{mol} \) collagen/L in the presence of catechin (50 and 100 \( \mu \text{mol} \)/L), quercetin (10 and 20 \( \mu \text{mol} \)/L), and catechin (25 \( \mu \text{mol} \)/L) plus quercetin (5 \( \mu \text{mol} \)/L)
is reported in Figure 5. Catechin or quercetin alone inhibited platelet adhesion to collagen, which was almost completely suppressed by 100 μmol/L catechin and 20 μmol/L quercetin. Incubation of platelets with 25 μmol catechin/L plus 5 μmol quercetin/L inhibited platelet adhesion significantly by 85%.

DISCUSSION

Previous studies showed that quercetin (40–100 μmol/L) and catechin (100–420 μmol/L) inhibited platelet aggregation in vitro (5–7). We confirmed this effect using 50 and 100 μmol catechin/L. Lower concentrations of quercetin were sufficient to inhibit platelet function; the inhibitory effect achieved with 20 μmol quercetin/L was the same as that observed with 100 μmol catechin/L. The disagreement with the data reported in the literature might be dependent on our experimental procedure. Before the addition of the agonist, platelets were incubated with flavonoids for 30 min, an incubation time that was much longer than that used previously (10 min) in studies of the effects of flavonoids on platelet function.

It has been suggested that the effect of flavonoids on platelet aggregation is dependent on the inhibition of the cyclooxygenase pathway, resulting from the inhibitory effect of flavonoids on the platelet formation of thromboxane A2, a potent aggregating and vasoconstricting agent (5–7). It was suggested that the underlying mechanism resides in the antioxidant activity of flavonoids, but this hypothesis has never been investigated. We showed recently that collagen-induced platelet aggregation is associated with a burst of
hydrogen peroxide that, in turn, contributes to the activation of platelet function through calcium mobilization and inositol pathway activation (19). Therefore, this experimental model is considered ideal to assess whether flavonoids behave as antioxidant agents when incubated with platelets. We showed that both quercetin and catechin significantly inhibited the release of platelet hydrogen peroxide elicited by collagen, with almost complete suppression when the 2 flavonoids were used in combination. An apparent discrepancy in our findings is that the inhibition of collagen-induced hydrogen peroxide release by quercetin or catechin, both alone and in combination, was more marked than was the inhibition of platelet aggregation. This is likely dependent on the fact that hydrogen peroxide represents only one pathway through which collagen induces platelet aggregation. On the basis of the above-reported antioxidant properties, it is presumed that platelet aggregation could not be completely suppressed by flavonoids.

According to a previous study showing that hydrogen peroxide contributes to activating the phosphoinositide pathway, quercetin and catechin alone or in combination significantly inhibited calcium mobilization and IP₃ formation because of their ability to quench hydrogen peroxide. Therefore, these findings indicate that quercetin and catechin inhibit platelet function by virtue of antioxidant activity. This hypothesis is corroborated by a previous study (20) in which we tested the effect of another antioxidant, vitamin E, in the same experimental model. Vitamin E significantly inhibited platelet activation at a concentration of 5 μmol/L, which is similar to the concentrations of quercetin and catechin used in the present study. However, a more precise comparison between vitamin E and flavonoids could not be done because lower concentrations of vitamin E were not investigated.

The close relation between red wine consumption and the inhibition of platelet function has been observed in several experimental studies. In fact, intragastric administration of 4.0 mL red wine/kg body wt completely suppressed platelet activation in a canine model of coronary stenosis (3). Even though flavonoid concentrations in peripheral blood were not measured after red wine administration, further study on the same experimental model showed that flavonoids inhibited platelet activation, thus suggesting their possible involvement in the inhibition of platelet function (4). We cannot be certain that the concentrations used in our experiments correspond to those observed in human circulation after red wine consumption because there are no data concerning this point. However, with other sources of flavonoids, such as onions and tea, the plasma flavonoid concentrations ranged from 0.6 to 13 μmol/L (19–22), which are similar to those achieved by the combination of quercetin and catechin in the present study. In our experiments, the incubation of platelets with 5 μmol quercetin/L plus 20 μmol catechin/L, which singly had no effect on platelet function, was associated with a significant inhibition of platelet activation. Note that even though the stimulation was carried out with high concentrations of collagen (8–20 mg/L), which were technically necessary to detect calcium mobilization and IP₃ production (19), the combination of quercetin and catechin inhibited platelet function.

The combination of catechin and quercetin elicited even more profound effects on platelet adhesion, which was almost completely suppressed when platelets were treated with the 2 flavonoids. To the best of our knowledge, this is the first evidence that flavonoids inhibit platelet adhesion. Because of the biological importance of platelet adhesion to collagen in the initiation and progression of the atherosclerotic process (23), this finding may be important to further understand the relation between red wine, flavonoids, and cardiovascular disease.

In conclusion, our study showed for the first time that flavonoids such as quercetin and catechin act synergistically to inhibit platelet adhesion to collagen and collagen-induced platelet aggregation. These data further contribute to the understanding of the relation between moderate consumption of red wine and a decreased risk of cardiovascular disease.

REFERENCES