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

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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 resveretrol, 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 A2 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.

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MATERIALS AND METHODS

Materials

\(^{35}\text{Pi}\) and \([^{3}H]\)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 \(\mu\)mol Fura 2-AM/L, 40 \(\mu\)mol DCFH-DA/L, 7.4 GBq (2 Ci) \(^{35}\text{Pi}\)/L, or 3.7 MBq (1 mCi) \([^{3}H]\)oleic acid/L. Platelets were washed by gel filtration on Sepharose 2B by using Ca\(^{2+}\)-free Tyrode’s buffer (134 mmol NaCl/L, 2.9 mmol KCl/L, 0.34 mmol \(\text{Na}_{2}\text{HPO}_4)/L, and 2 mmol MgCl\(_2)/L) containing 0.2% bovine serum albumin, 5 mmol glucose/L, and 10 mmol HEPES/L, pH 7.35. Gel-filtered platelets (GFPs) were adjusted to a final concentration of 2–4 mg/L.

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 increased 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; 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 \(\mu\)mol catechin/L inhibited \(\approx 75%\) platelet aggregation induced by 2 mg collagen/L and inhibited \(\approx 39%\) platelet aggregation induced by 4 mg collagen/L. In platelets treated with 20 \(\mu\)mol 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 \(\mu\)mol catechin/L and 5 \(\mu\)mol 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 \(\mu\)mol catechin/L and 20 \(\mu\)mol quercetin/L significantly decreased calcium mobilization by 71% and 65%, respectively.
The incubation of platelets with 25 μmol catechin/L plus 5 μmol 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**

[^32P]IP₃ production in collagen-stimulated platelets was inhibited by catechin and quercetin: 10 mg collagen/L, 100 μmol catechin/L, and 20 μmol quercetin/L significantly decreased IP₃ production by 50% and 93%, respectively. The incubation of platelets with 25 μmol catechin/L plus 5 μmol quercetin/L significantly inhibited IP₃ 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 μmol collagen/L in the presence of catechin (50 and 100 μmol/L), quercetin (10 and 20 μmol/L), and catechin (25 μmol/L) plus quercetin (5 μmol/L)
is reported in Figure 5. Catechin or quercetin alone inhibited platelet adhesion to collagen, which was almost completely suppressed by 100 μmol catechin/L and 20 μmol quercetin/L. 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/L, quercetin (Q; 10 and 20 μmol/L), or Cat (25 μmol/L) + Q (5 μmol/L). n = 5 experiments. *Significantly different from collagen alone: *P < 0.05, #P < 0.01.

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 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.
quercetin and catechin alone or in combination significantly contribute to activating the phosphoinositol pathway, aggregation could not be completely suppressed by flavonoids. On the basis of the above-reported antioxidant properties, it is presumed that platelet function through calcium mobilization and inositol pathway activation (19). Therefore, this experimental model is 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 the activation of platelet function through calcium mobilization and inositol pathway activation (19), the combination of catechin and quercetin elicited even more profound effects on platelet aggregation, 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