Effects of the flavonoids quercetin and apigenin on hemostasis in healthy volunteers: results from an in vitro and a dietary supplement study

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ABSTRACT Intake of dietary flavonols and flavones was inversely associated with risk for cardiovascular disease in several epidemiologic studies. This may have been due to effects on hemostasis because flavonoids have been reported to inhibit platelet aggregation in vitro. We indeed found that 2500 μmol/L of the flavonol quercetin and the flavone apigenin significantly inhibited collagen- and ADP-induced aggregation in platelet-rich plasma and washed platelets by ~80–97%. However, lower concentrations, such as might occur in vivo, had no effect. To test this in vivo we fed 18 healthy volunteers 220 g onions/d providing 114 mg quercetin/d, 5 g dried parsley/d providing 84 mg apigenin/d, or a placebo for 7 d each in a randomized crossover experiment with each treatment period lasting 2 wk. Onion consumption raised mean plasma quercetin concentrations to 1.5 μmol/L; plasma apigenin could not be measured. No significant effects of onions or parsley were found on platelet aggregation, thromboxane B2 production, factor VII, or other hemostatic variables. We conclude that the antiaggregatory effects of flavonoids seen in vitro are due to concentrations that cannot be attained in vivo. Effects of dietary flavonols and flavones on cardiovascular risk are possibly not mediated by hemostatic variables. Am J Clin Nutr 1998;67:255–62.

KEY WORDS Apigenin, diet, factor VII, fibrinogen, flavonol, hemostasis, humans, plasminogen activator inhibitor 1, PAI-1, plasminogen, platelet aggregation, quercetin, thromboxane, in vitro study, onions, parsley

INTRODUCTION
Flavonoids are polyphenolic compounds that occur ubiquitously in plant foods. Flavonols and flavones are subclasses of flavonoids (Figure 1) (1–4). In 1992 the average daily intake of the flavonol quercetin, kaempferol, and myricetin from the Dutch diet was 16, 4, and 1 mg, respectively; the average intake of the flavonols quercetin, kaempferol, and myricetin from the Dutch diet was 16, 4, and 1 mg, respectively; the average intake of the flavonols quercetin, kaempferol, and myricetin from the Dutch diet was 16, 4, and 1 mg, respectively; the average intake of the flavonols quercetin, kaempferol, and myricetin from the Dutch diet was 16, 4, and 1 mg, respectively; the average intake of the flavonols quercetin, kaempferol, and myricetin from the Dutch diet was 16, 4, and 1 mg, respectively. Intake of dietary flavonoids was 16, 4, and 1 mg, respectively; the average intake of the flavonoids quercetin and the flavone apigenin significantly inhibited collagen- and ADP-induced aggregation in platelet-rich plasma and washed platelets by ~80–97%. However, lower concentrations, such as might occur in vivo, had no effect. To test this in vivo we fed 18 healthy volunteers 220 g onions/d providing 114 mg quercetin/d, 5 g dried parsley/d providing 84 mg apigenin/d, or a placebo for 7 d each in a randomized crossover experiment with each treatment period lasting 2 wk. Onion consumption raised mean plasma quercetin concentrations to 1.5 μmol/L; plasma apigenin could not be measured. No significant effects of onions or parsley were found on platelet aggregation, thromboxane B2 production, factor VII, or other hemostatic variables. We conclude that the antiaggregatory effects of flavonoids seen in vitro are due to concentrations that cannot be attained in vivo. Effects of dietary flavonols and flavones on cardiovascular risk are possibly not mediated by hemostatic variables. Am J Clin Nutr 1998;67:255–62.

Flavonoids may also affect the activity or the concentration of plasma coagulation or fibrinolysis factors such as fibrinogen, factor VII, and plasminogen (24–26). The formation of a thrombus in atherosclerotic coronary arteries gives rise to acute ischemic heart disease, and coagulation and fibrinolysis factors play a key role in the control of thrombus formation (27, 28). Several studies indeed showed that plasma fibrinogen concentration is an independent risk factor for ischemic heart disease (29–31). Factor VII and plasminogen activity were also associated with ischemic heart disease risk (30) and plasma plasminogen activator inhibitor 1 (PAI-1) activity was associated with increased risks of myocardial (re)infarction (31, 32).

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We carried out both an in vitro study and a dietary supplement study in healthy volunteers. In the in vitro study we investigated whether a test tube addition of flavonoids in the estimated physiological range (0–2.5 μmol/L) inhibited in vitro platelet aggregation, and we included unphysiologically high concentrations of flavonoids (>2.5 μmol/L) to enable comparisons with published studies. In the dietary study we examined whether administration of foods rich in flavonoids affected platelet aggregation and coagulation and fibrinolysis factors in healthy volunteers.

SUBJECTS AND METHODS

Both protocols were approved by the Medical Ethics Committee of the Department of Human Nutrition of Wageningen Agricultural University and were fully explained to the participants, who gave their written, informed consent. Participants were all nonsmokers. The in vitro study was carried out in the summer of 1995 and the dietary supplement study from October until December 1995.

In vitro study

Subjects

Four men from the Department of Human Biology, Maastricht University, aged 24, 29, 35, and 47 y served as blood donors. All were healthy according to a medical questionnaire and ate an ordinary Western diet. None of the blood donors took any medication for 2 wk preceding the study until the end of the measurements.

Methods

We investigated the effects on platelet aggregation of six concentrations (0, 0.25, 2.5, 25, 250, and 2500 μmol/L) of pure quercetin-3-glucoside (Apin Chemicals LTD, Abingdon Oxon, United Kingdom); quercetin aglycone, ie, quercetin without a sugar moiety (quercetin dihydrate, no. 75670; Fluka Chemika, Buchs, Germany); apigenin aglycone (no. 10798; Fluka Chemika, Meppel, Netherlands); and catechin [(+)-catechin hydrate; Fluka Chemika, Meppel, Netherlands]. It was not possible to study in vitro effects of apigenin glycosides because these glycosides are not available commercially. Effects were studied in both platelet-rich plasma and washed platelets to exclude possible effects of plasma factors on aggregation. Effects were not studied in whole blood because whole blood is only stable for ~30 min.

Indomethacin (I-7278; Sigma Chemical Co, St Louis), a specific inhibitor of the enzyme cyclooxygenase, was used as a positive control. Effects of final indomethacin concentrations of 0, 0.1, 1, 10, 100, and 1000 μmol/L on in vitro platelet aggregation in platelet-rich plasma and washed platelets were tested by using citrated blood from one of the donors. Aggregations in platelet-rich plasma were stimulated with final concentrations of 2 mg collagen/L (Collagen Horm, München, Germany) or 2.5 μmol ADP/L (A6521; Sigma Chemical Co). Aggregations in washed platelets were induced by 2.5 mg collagen/L or 18 μmol ADP/L.

Because polyphenols are known to bind proteins, catechin—a flavonoid with a high protein binding capacity (33)—served as a control for nonspecific effects. Each donor gave blood four times within a period of 1–6 wk. Effects of apigenin, quercetin, quercetin-3-glucoside, and catechin were tested in random order for all four donors. Effects of the six different concentrations of each compound were tested on the same day in random order.

After the participants had fasted overnight, free-flowing venous blood was sampled without stasis (Strauss Kanüle, 1.2-mm syringe; Luer, Wächterbach, Germany) with the subject in a supine position. The first 3 mL blood was discarded. Blood was collected into tubes prefilled with a sodium citrate solution (final concentration: 10.9 mmol/L, pH 7.3; Merck BV, Amsterdam). Platelet-rich plasma was centrifuged at 150–160 × g for 15 min at room temperature and diluted with autologous platelet-poor plasma to a final concentration of 185 × 10^9 platelets/L. Washed platelets were prepared by mixing 5.8 mL of 80 mmol trisodium citrate/L, 52 mmol citric acid/L, and 183 mmol glucose [acid citrate dextrose (ACD)]/L with 29.2 mL blood. This solution was centrifuged for 15 min at 160 × g at room temperature and platelet-poor plasma was removed. Subsequently, 25 mL platelet-rich plasma was mixed with 1 mL ACD, centrifuged for 15 min at 610 × g at room temperature, and the platelet pellet was resuspended in 2 mL HEPES buffer (pH 6.6; 136 mmol NaCl/L, 27 mmol KC1/L, 10 mmol HEPES/L, 1 mmol MgCl2, 6H2O/L, 1 g glucose/L, and 1 g bovine serum albumin/L); HEPES buffer was added to a total volume of 30 mL, and 0.07 L ACD/L buffer was added. The suspension was centrifuged at 610 × g for 15 min at room temperature, and the platelet pellet was resuspended in HEPES buffer (pH 7.45). Glucose and bovine serum albumin were added to the buffer just before use.

After the first blood donation, optimal doses of the stimuli collagen and ADP were determined for each person by using 5 μL blank solvent dimethylsulfoxide (DMSO; Fluka Chemika, Meppel, Netherlands) instead of a flavonoid solution, under conditions as described below. The optimal stimulus doses, defined as the final concentrations leading to a maximal aggregation of 65%, were as follows for the aggregations in platelet-rich plasma of the four donors: 2.0, 4.0, 2.0, and 4.0 mg collagen/L and 9.0, 5.0, 2.5, and 2.5 μmol ADP/L. The optimal doses for aggregation in washed platelets of the four donors were 7.5, 7.5, 2.5, and 7.5 mg collagen/L and 18.0, 18.0, 18.0, and 6.0 μmol ADP/L, respectively. These doses were used throughout.

For each aggregation measurement, 400 μL diluted platelet-rich plasma or washed platelets were incubated in an aggregometer at 37 °C at 1000 rpm (Chronolog Corporation, Havertown, PA). Flavonoids were dissolved in DMSO and 5 μL was
added to the platelets to produce final concentrations of 0, 0.25, 2.5, 25, 250, and 2500 μmol/L. Ten microliters of collagen suspension or ADP was added exactly 10 min after addition of the flavonoid. We added fibrinogen (final concentration: 0.5 g/L, fraction 1, type 4 bovine plasma; Sigma Chemie, Brunsswig, Amsterdam) during the ADP-induced aggregation of washed platelets. The change in percentage of transmitted light was monitored continuously for 7 min. Light transmitted was set at 100% for platelet-poor plasma and HEPES buffer and at 0% for platelet-rich plasma and the washed platelet suspension.

Aggregation measurements were completed within 2 h after blood sampling. We found in earlier studies (unpublished observations, 1995) that platelets were stable during this period. Platelets were always handled in plastic material and at room temperature. Maximal aggregation was calculated for all measurements. For ADP-induced aggregations, maximal aggregation of the first wave was used as the outcome variable. Maximal aggregation on 5 μL solvent DMSO using optimal individual stimulus concentrations was chosen as 100%. Means and SDs of the maximal aggregation values were calculated for each flavonoid concentration as the means and SDs of the four donors.

Statistics

Within-person effects of the addition of flavonoids to the platelet-rich plasma compared with no flavonoid addition were calculated for each flavonoid concentration tested. We then calculated the mean effects (n = 4) and the 95% CIs of these effects. For platelet-rich plasma and washed platelets the power to detect a difference between two aggregation measurements, by using a within person CV of 11%, was 90% to detect a difference of 18% (α = 0.05).

Dietary supplement study

Subjects

Ten men and 12 women were recruited through announcements in the University newspaper and posters in student dormitories. One man was excluded because of elevated alanine aminotransferase and γ-glutamyltransferase concentrations and one woman withdrew on her own accord. From the remaining 20 subjects, 9 men and 9 women were selected by drawing lots. One man dropped out during the first week of the study for personal reasons unrelated to the study. A woman who had participated in the screening replaced him; she started at day 7 of the study. There were no significant differences in the outcomes of the dependent variables when the data of this woman were excluded (data not shown). All 18 volunteers successfully completed the study.

Participants were healthy on the basis of a medical questionnaire and had normal values for urinary protein and glucose, hematocrit, hemoglobin, white blood cell and platelet counts, mean red cell volume, plasma alanine aminotransferase, plasma γ-glutamyltransferase, serum creatinine, prothrombin, and activated partial thromboplastin time. The mean (± SD) age of the subjects was 25 ± 8 y and body mass index (kg/m²) was 22 ± 1.

Design

We investigated the effects of daily consumption of flavonoid-rich dietary supplements on indexes for hemostasis in a randomized, placebo-controlled, multiple crossover study involving three treatments, each treatment period lasting 2 wk. All subjects participated simultaneously. Participants consumed supplements daily during weeks 2, 4, and 6. Week 1 served as the run-in period and weeks 3 and 5 as washout periods. Supplements were given in random order.

Supplements

Supplements consisted of 400 g bouillon, to which was added either 220 g cooked yellow onions (Favorit MSP, class II 60–80 mm; Luctor, Dronten, Netherlands), 4.9 g dried Western European parsley (Verstegen Specerijen, Rotterdam, Netherlands), or nothing as a placebo. Onions, parsley, and bouillon powder (Maggi Bouillonkorrels; Nestlé Foodservice Catering, Amsterdam) were bought in one batch just before the study and stored in the dark at 4 °C.

The onions were thinly peeled 1 wk before the study started, cut into pieces of 8 × 8 × 8 mm in a blender (Robot-Coupe SA, Montceau-en-Bourgogne, France), heated in portions of 600 g in a microwave oven for 5 min at 800 W or 7 min at 500 W, mixed, and heated for another 4 min at 800 W or 5 min at 500 W. Portions of 220 g were weighed on a digital scale to a precision of 0.01 g (model 1203 MP; Sartorius, Gottingen, Germany) and stored at −20 °C. Onions were thawed in the dark at room temperature overnight before consumption. Before the study started the parsley was mixed, weighed out in portions of 4.9 g, and stored in the dark at room temperature until consumption. Bouillon was prepared each day before consumption by using 16.7 g bouillon powder/kg boiling water; 400-g portions were weighed out and stored in the dark at 4 °C. Duplicate portions of the supplements were prepared daily and stored at −20 °C until analyzed (35). The onion supplement contained 114 ± 3 mg quercetin (377 ± 10 μmol; n = 15) and the placebo 0.015 ± 0.004 mg quercetin (0.05 ± 0.01 μmol; n = 6). The parsley supplement contained 84 ± 6 mg apigenin (n = 15).

During weeks 2, 4, and 6, fasted subjects came to the department daily between 0730 and 0900 on working days and between 0800 and 1000 on weekend days to consume their supplements. Parsley and onions were mixed with the bouillon just before consumption. The onion soup was heated for 6 min at 800 W or for 7 min at 500 W; the parsley soup and placebo bouillon were heated for 4.5 min at 500 W or 3.5 min at 800 W. Participants were not allowed to eat or drink anything except mineral water until 2 h after consumption of their supplements.

Food consumption, physical activity, and medication

Participants were urged not to consume any fruit or vegetables containing > 15 mg quercetin or apigenin/kg (eg, apples, endive, beans, broccoli, celery, cherries, cloves, grapes, leeks, onions, parsley, and tomatoes), any beverages containing > 4 mg quercetin or apigenin/L (eg, tea and wine) (35–38), or any fatty fish. Subjects were asked to maintain their normal eating and drinking habits and physical activity levels during the study as much as possible, given the restrictions mentioned above.

Subjects were instructed to avoid taking traditional and homeopathic medicines and vitamin and mineral supplements from 1 mo preceding the study until the end of the study. Use of oral contraceptives was permitted. Participants were supplied with acetaminophen (paracetamol; Samenwerkende Apothekers, Utrecht, Netherlands), which could be used for pain relief. Subjects were urged to record health complaints, medications taken, and any deviations from their normal physical activity and dietary habits in
a diary. We determined the body weights of the participants and checked the diaries weekly at days 1, 8, 15, 22, 29, 36, and 42.

An experienced dietitian trained one of the human nutrition students involved in the study to determine food intake. The food intake of each participant was measured on 1 d in week 2, 1 d in week 4, and 1 d in week 6 by using 24-h dietary recalls (39); 29% of all recalls were from weekend days whereas 71% were from weekdays. The dietitian and student were responsible for collecting food intake data. Recalls of a particular subject were handled by the same person during the whole study. We regularly checked the between-interviewer variation, which turned out to be negligible.

The habitual energy intake of the subjects was 9.3 ± 2.7 MJ/d (2214 ± 642 kcal/d), of which 31 ± 9% was provided by fat, 14 ± 3% by protein, 53 ± 10% by carbohydrate, and 1 ± 3% by alcohol, with no changes during the study. Mean body weight decreased by 0.3 ± 1.4 kg (NS) during the study. Subjects did not consume any fatty fish. There was no evidence from the dietary recall data of changes in physical activity patterns or any deviations that might have affected the results. One subject took iron tablets (Ferrofumarat, three 200-mg tablets/d; FNA, The Hague) from day 33 until the end of the study because of low hemoglobin concentrations. Subjects took no medications during the study, except for the acetaminophen supplied by us. All participants denied having taken acetylsalicylic acid from 1 mo preceding the study until the end of the study.

Blood sampling

Blood samples were taken on days 14, 28, and 42 as described above. About 25 mL blood was drawn 90 min after subjects had consumed the supplement. Subjects were in a supine position for 20 min before until the end of venipuncture. Blood was drawn into tubes containing a final concentration of 10.9 mmol sodium citrate/L. Samples for coagulation and fibrinolysis measurements were put on ice immediately.

Analyses

Immediately after venipuncture 1 mL citrated blood was incubated in a prewarmed aggregometer (37 °C) and stirred at 1000 rpm. Exactly 5 min later 10 μL collagen suspension (final concentration: 2 mg/L) was added and whole-blood aggregation was recorded for 10 min on a computerized system with an impedance method. Maximal aggregation was measured in whole blood to study effects of the supplements on platelet aggregation under most physiologic conditions. Platelet-rich plasma containing 250 × 10^6 platelets/L was prepared as described previously (40). Some diluted platelet-rich plasma was frozen in fluid nitrogen and stored at −80 °C for determination of flavonoid concentrations. HPLC separation was used combined with fluorescence detection for determination of quercetin (41) and with ultraviolet detection for apigenin concentrations (35). The interassay CV for quercetin in plasma was 4% and the intraassay CV was 4%.

For each aggregation, 400 μL platelet-rich plasma was incubated in a prewarmed aggregometer and stirred at 1000 rpm. Exactly 5 min later, 10 μL collagen (final concentration: 2.0 mg/L) or ADP (final concentration: 1.5 and 3.0 μmol/L) was added to induce aggregation. The change in percentage of transmittance was monitored continuously for 10 min and maximal aggregation was calculated as described for the in vitro study. Aggregations in platelet-rich plasma were carried out to compare our results with the results of others and with the results of our in vitro data. Maximally stimulated thromboxane B2 production in platelet-rich plasma was measured as described earlier (40) as a specific measure of active cyclooxygenase present. All samples of one subject were analyzed within one run.

Immediately after blood sampling, plasma for measurements of coagulation and fibrinolysis indexes was separated by centrifugation at 1500 × g for 20 min at 4 °C. It was divided into aliquots, snap-frozen in liquid nitrogen, and stored at −80 °C. Factor VII and plasminogen activity were determined by a laser-nephelometric centrifugal ACL-200 analyzer (Instrument Laboratory, Milano, Italy). Factor VII clotting time was determined in a standard one-stage assay: plasma samples and factor VII–deficient plasma were mixed, the clotting process was initiated, and clotting time was measured by using PT-fibrinogen and thromboplastin, both from the Instrumentation Laboratory (IJsselstein, Netherlands), and factor VII from Organon Technica (Oss, Netherlands). Plasminogen activity was determined according to the test manufacturer’s instructions by using COATEST antiplasmin and streptokinase from Chromogenix (Amsterdam). Blood for a normal plasma pool was donated by 40 healthy volunteers. Factor VII and plasminogen results were expressed in percentages relative to values for this pool. Standards for factor VII and plasminogen measurements were derived from BIOPOL (hemostasis reference plasma; Kordia, Leiden, Netherlands). Plasma fibrinogen concentrations were determined with an STA II coagulation analyzer (STA-fibrinogen; Diagnostica Stago, Boehringer Mannheim, Mannheim, Germany); a fixed surplus of thrombin was added to diluted platelet-poor plasma samples and the clotting time of a series of dilutions of a human plasma pool with a known fibrinogen concentration was measured. Standards for fibrinogen measurements were derived from Boehringer Mannheim (STA-Preci clot I and II). PAI-1 activity was measured with a chromogenic assay (Spectrolyse/mL PAI; Biopool, Umea, Sweden). Plasminogen and factor VII measurements were done in duplicate; fibrinogen and PAI-1 measurements were done once.

Statistics

We checked the data for normality using residual analysis (42). Effects of onions and parsley were analyzed by using the general linear model (analysis of variance, fixed effect) of the Statistical Analysis System (SAS, Cary, NC). Student’s t-test was used to check for significant differences between treatment groups. The Statistical Analysis System (SAS, Cary, NC) was used to analyze data. For blood pressure differences, a linear mixed-effects model was used. Differences were regarded as significant if p < 0.05. Statistical analysis of data was done with SAS, version 9.1 for Windows (SAS Institute, Cary, NC). The data are represented as mean ± SD.

RESULTS

In vitro study

Indomethacin at a concentration of 1 μmol/L inhibited maximal collagen-induced aggregation in human platelet-rich plasma by 67% and in washed platelets by 74%; 1000 μmol/L inhibited...
aggregation in platelet-rich plasma by 98% and in washed platelets by 82% (data not shown). ADP-induced aggregation was hardly affected.

Catechin inhibited collagen- and ADP-induced aggregation in platelet-rich plasma significantly only at a concentration of 2500 µmol/L (Figures 2 and 3): average maximum collagen-induced aggregation was inhibited by 59 ± 25% (95% CI: 19%, 99%) and ADP-induced aggregation by 26 ± 17% (95% CI: 1%, 53%). Results in washed platelets were similar to results found in platelet-rich plasma (data not shown).

Apigenin at a concentration of 2.5 µmol/L inhibited collagen-induced aggregation in platelet-rich plasma by 24 ± 34% (NS) and ADP-induced aggregation by 22 ± 33% (NS). Concentrations of 2500 µmol/L significantly inhibited collagen-induced aggregation by 91 ± 4% (95% CI: 85%, 97%) and ADP-induced aggregation by 80 ± 13% (95% CI: 59%, 101%) (Figures 2 and 3).

Concentrations of 0.25–2500 µmol/L of the flavonol quercetin significantly inhibited collagen-induced aggregation by 95 ± 4% (95% CI: 89%, 101%) and ADP-induced aggregation by 97 ± 4% (95% CI: 91%, 103%). Lower concentrations of quercetin were ineffective (Figures 2 and 3).

In platelet-rich plasma, 2500 µmol/L of the flavonol quercetin significantly inhibited collagen-induced aggregation by 95 ± 4% (95% CI: 89%, 101%) and ADP-induced aggregation by 97 ± 4% (95% CI: 91%, 103%). Lower concentrations of quercetin were ineffective (Figures 2 and 3). Effects on aggregation in washed platelets were comparable with those in platelet-rich plasma (Figures 2 and 3).

Dietary supplement study

No adverse reactions to the supplements were reported, although some subjects had difficulties in consuming the amount of onions all at once. One subject consumed only part of the onion supplement on one occasion; the leftovers contained 29 mg quercetin. Subjects consumed negligible amounts of flavonol- or flavone-rich products during the study.

Concentrations of quercetin in platelet-rich plasma were 1.48 ± 0.39 µmol/L (447 ± 117 ng/mL) 90 min after consumption of onions and 0.02 ± 0.01 µmol/L (5 ± 4 ng/mL) after placebo. Apigenin concentrations in platelet-rich plasma were all below the limit of detection of 1.1 µmol/L, or 330 ng/mL.

Daily consumption of 220 g cooked onions or 4.9 g dried parsley for 7 d did not significantly affect collagen-induced platelet aggregation in whole blood or platelet-rich plasma; ADP-induced platelet aggregation in platelet-rich plasma; thromboxane B2 production in platelet-rich plasma; platelet number; factor VII, plasminogen, and PAI-1 activity; or fibrinogen concentrations (Table 1). There were no treatment-sequence or time effects.

DISCUSSION

The in vitro study showed that a test tube addition of 0.25–250 µmol/L of apigenin, catechin, quercetin, or quercetin-3-glucoside did not inhibit collagen- or ADP-induced aggregation in human platelets. Quercetin, apigenin, and catechin concentrations of 2500 µmol/L inhibited platelet aggregation significantly in vitro, whereas quercetin-3-glucoside still had no significant effect. The dietary supplement study showed that administration of large amounts of foods rich in apigenin or quercetin glucosides did not affect platelet aggregation or other hemostatic variables in healthy volunteers.

In vitro study

We carried out highly standardized aggregation measurements in both studies. Published in vitro studies were not always strictly standardized as to platelet number and optimal stimulus concentration for each donor, and the experimental conditions varied from one study to another, which probably explains the large variation in outcome (14–21).

To check the validity of the in vitro aggregation measurements, indomethacin was used as a positive and catechin as a negative control. As expected, indomethacin—a specific inhibitor of cyclooxygenase (44)—inhibited in vitro platelet aggregation induced by collagen but not by ADP. We therefore conclude that our assay could detect specific inhibiting effects on cyclooxygenase activity in both platelet-rich plasma and washed platelets. The in vitro study showed that concentrations of 0.25 and 2.5

FIGURE 2. Relation between flavonoid concentration added to platelet-rich plasma in vitro and maximal platelet aggregation induced with collagen (n = 4). ○, catechin; ●, quercetin; ▼, quercetin-3-glucoside; □, apigenin.
μmol catechin/L, which has a high affinity for proteins, did not affect collagen- or ADP-induced platelet aggregation, although unphysiologically high concentrations (2500 μmol/L) did (Figures 2 and 3). The latter may be a nonspecific inhibiting effect because flavonoids readily bind to proteins (33). Thus, it is not likely that nonspecific protein binding would explain the possible effects of flavonoids at physiologic concentrations.

**Dietary supplement study**

Values of the hemostatic variables measured in the dietary supplement study after treatment with placebo were in the normal range for healthy volunteers (Table 1). The design of the dietary supplement study was not optimal: a controlled feeding study would have reduced potential confounding from foods eaten during the study. However, data from the diaries, body weights, and 24-h recalls did not reveal any confounding effects. Furthermore, all 18 subjects ate the supplements under our supervision and plasma quercetin concentrations after treatment with placebo were in the normal range for healthy volunteers (Table 1). The design of the dietary supplement study after treatment with placebo were in the normal range for healthy volunteers (Table 1). The design of the dietary supplement study after treatment with placebo were in the normal range for healthy volunteers (Table 1). The design of the dietary supplement study after treatment with placebo were in the normal range for healthy volunteers (Table 1).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Placebo</th>
<th>Effect (treatment—placebo)</th>
<th>Onions</th>
<th>Parsley</th>
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<tbody>
<tr>
<td>Factor VII activity (n = 18) (%)</td>
<td>92 ± 25</td>
<td>−4 ± 13 (−10, 2)</td>
<td>−1 ± 2 (−2, 0)</td>
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<td>Fibrinogen (n = 18) (g/L)</td>
<td>2.4 ± 0.6</td>
<td>0.1 ± 0.7 (−0.2, 0.4)</td>
<td>0.2 ± 0.6 (−0.1, 0.5)</td>
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<tr>
<td>Plasminogen activity (n = 17) (%)</td>
<td>87 ± 19</td>
<td>1 ± 8 (−3, 5)</td>
<td>0 ± 6 (−3, 5)</td>
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<td>PAI-1 activity (n = 17) (kU/L)</td>
<td>4.35 ± 3.54</td>
<td>−0.90 ± 3.17 (−2.66, 0.86)</td>
<td>0.31 ± 3.97 (−1.73, 2.35)</td>
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<tr>
<td>B2 production in platelet-rich plasma (n = 18) (nmol/10¹¹ platelets)</td>
<td>2502 ± 593</td>
<td>−122 ± 438 (−340, 96)</td>
<td>−131 ± 503 (−381, 119)</td>
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<td>Maximal collagen-induced aggregation in whole blood (final 2 mg/L; n = 18) (%)</td>
<td>14 ± 4</td>
<td>−0.6 ± 5.2 (−3.2, 2.0)</td>
<td>−0.1 ± 5.7 (−2.9, 2.7)</td>
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<td>Maximal collagen-induced aggregation in platelet-rich plasma (final 2 μg/L; n = 13) (%)</td>
<td>84 ± 13</td>
<td>−6.4 ± 14.5 (−15.2, 2.4)</td>
<td>−10.5 ± 27.4 (−27.1, 6.1)</td>
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<tr>
<td>Maximal ADP-induced aggregation in platelet-rich plasma (final 1.5 μmol/L; n = 18) (%)</td>
<td>23 ± 8</td>
<td>7 ± 20 (−4, 18)</td>
<td>6 ± 19 (−4, 16)</td>
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<td>Maximal ADP-induced aggregation (%) in platelet-rich plasma (final 3 μmol/L; n = 16) (%)</td>
<td>57 ± 28</td>
<td>−8 ± 22 (−20, 4)</td>
<td>−6 ± 26 (−20, 8)</td>
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<td>Platelet number (n = 18) (×10¹²/L)</td>
<td>235 ± 47</td>
<td>0 ± 28 (−14, 14)</td>
<td>9 ± 25 (−3, 21)</td>
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7 x ± SD; 95% CIs in parentheses.

3 Quercetin concentration in platelet-rich plasma: 0.02 ± 0.01 μmol/L.

1 Quercetin concentration in platelet-rich plasma: 1.48 ± 0.39 μmol/L.

4 Apigenin concentrations in platelet-rich plasma were lower than the limit of detection (1.1 μmol/L).

5 n = 15.

6 n = 17.

7 First wave.

9 n = 16.

Comparison with results of earlier studies

Gryglewski et al (17) reported stimulation of cyclooxygenase activity after adding quercetin or rutin to ram seminal vesicle microsomes. In contrast, results of other structure-function studies suggest that flavones such as apigenin are strong inhibitors and flavonols such as quercetin are moderate inhibitors of cyclooxygenase. Those studies also suggested that glycosylated compounds are less potent inhibitors of cyclooxygenase than their aglycones (14, 21). A major drawback of in vitro studies done earlier is that only the effects of unphysiologically high flavonoid contents (> 10 μmol/L) were studied (14–21). It was shown recently that plasma peak quercetin concentrations in humans were 0.6 μmol/L after consumption of 64 mg quercetin from onions (23). Although no data on apigenin absorption are available, our data showed that plasma peak apigenin concentrations in humans were <1.1 μmol/L. We found that the flavone apigenin and the flavonol quercetin significantly inhibited collagen-induced aggregation in vitro only at concentrations of 2500 μmol/L, whereas quercetin-3-glucoside, the form in which quercetin appears in foods and in which it is probably absorbed (22), did not affect collagen- or ADP-induced aggregation. The quercetin concentration in platelet-rich plasma after onion consumption was comparable with data reported earlier (23). The apigenin concentration in platelet-rich plasma could not be quantified because of the high limit of detection (1.1 μmol/L, or 330 ng/mL). Thus, it is unclear whether apigenin was absorbed. It is unlikely that apigenin absorption was inhibited by dietary proteins because the subjects were not allowed to eat or drink anything until 2 h after consumption of the supplements, and the supplements contained hardly any protein. The plasma concentration of quercetin suggests that these measures were adequate.
It is possible that plasma peak values and elimination curves of apigenin are different from quercetin (23) and that blood sampling at a different time point would reveal apigenin concentrations higher than the detection limit. In any case, if apigenin is absorbed, the prolonged supplementation would ensure interaction with the blood components. Epidemiologic studies showed that the inverse association between dietary flavonoid intake and ischemic heart disease risk was strongest with quercetin (9). Also, average daily dietary intake of quercetin was high (16 mg), compared with intake of apigenin (1 mg) in the Netherlands (5).

In accordance with our in vitro data, no effect of consumption of apigenin- or quercetin-rich foods was found on collagen-induced aggregation in whole blood and platelet-rich plasma, on ADP-induced aggregation in platelet-rich plasma, on thromboxane production, or on other hemostatic variables (Table 1). These findings indicate that daily consumption of large amounts of quercetin- or apigenin-rich foods may not be effective in inhibiting cyclooxygenase activity or platelet aggregation in human volunteers.

We think that the treatment periods of the dietary supplement study were long enough to detect possible effects on blood platelet function. Blood platelets have a mean lifetime of 7–10 d; therefore, treatment for 7 d with high doses of flavonoids should be sufficient to determine effects on platelet aggregation and thromboxane B2 production. We showed earlier in healthy volunteers that doses as small as 3 mg acetylsalicylic acid/d inhibited maximally stimulated platelet thromboxane production by 39 ± 8% (40). Therefore, we think that our study group was large enough to detect relevant effects on thromboxane production and platelet aggregation (Table 1). However, we might have missed small but biologically relevant effects (29–32) of the dietary flavonoids on the coagulation and fibrinolytic indexes measured (Table 1); this possibility should be examined in a larger study population.

The results of the present study agree with results from the dietary supplement study of Srivastava (45). They found no effects of consumption of 70 g raw onions/d for 7 d on platelet thromboxane production in five healthy volunteers: the mean effect of onion consumption on thromboxane production was 95 ± 756 nmol/L serum (n = 5). However, these authors could have missed biologically significant effects because of the small study sample, the low dose, and the large variation in the outcome variable. We did not find any effects in a larger group consuming 220 g onions/d. Hertog et al (5) showed that the average daily intake with the Dutch diet was 16 mg quercetin/d and 1 mg apigenin/d (5). In our dietary supplement study the participants consumed high doses of flavonoids: 114 mg quercetin/d and 84 mg apigenin/d. We think that consumption of higher doses is impractical in a normal mixed diet.

Conclusions

Suggestions for an antiaggregatory effect of flavonoids are based on the in vitro use of concentrations that cannot be attained in vivo by dietary consumption. Reported effects of dietary flavonoids on ischemic heart disease risk are possibly not mediated through collagen- or ADP-induced platelet aggregation or cyclooxygenase activity. We cannot exclude small biologically relevant effects of dietary flavonoids on known risk indicators for ischemic heart disease from the coagulation cascade or fibrinolytic system, which should be examined in a larger population.

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