The role of polyphenolic compounds in the diet as inhibitors of platelet function

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Platelets play a substantial role in cardiovascular disease, and for many years there has been a search for dietary components that are able to inhibit platelet function and therefore decrease the risk of cardiovascular disease. Platelets can be inhibited by alcohol, dietary fats and some antioxidants, including a group of compounds, the polyphenols, found in fruits and vegetables. A number of these compounds have been shown to inhibit platelet function both *in vitro* and *in vivo*. In the present study the effects of the hydroxycinnamates and the flavonoid quercetin on platelet activation and cell signalling *in vitro* were investigated. The hydroxycinnamates inhibited platelet function, although not at levels that can be achieved in human plasma by dietary intervention. However, quercetin inhibited platelet aggregation at levels lower than those previously reported. Quercetin was also found to inhibit intracellular Ca mobilisation and whole-cell tyrosine protein phosphorylation in platelets, which are both processes essential for platelet activation. The effect of polyphenols on platelet aggregation *in vivo* was also investigated. Twenty subjects followed a low-polyphenol diet for 3 d before and also during supplementation. All subjects were supplemented with a polyphenol-rich meal every lunchtime for 5 d. Platelet aggregation and plasma flavonols were measured at baseline and after 5 d of dietary supplementation. Total plasma flavonoids increased significantly after the dietary intervention period ($P = 0.001$). However, no significant changes in *ex vivo* platelet aggregation were observed. Further investigation of the effects of individual polyphenolic compounds on platelet function, both *in vitro* and *in vivo*, is required in order to elucidate their role in the relationship between diet and the risk of cardiovascular disease.

Dietary polyphenols: Hydroxycinnamates: Quercetin: Platelet function: Risk for cardiovascular disease

Cardiovascular disease encompasses a large number of diseases of the heart and vascular system a large proportion of which arise as a result of two pathophysiological processes, i.e. atherosclerosis and thrombosis. Platelets play a major role in thrombosis, and platelet aggregation is promoted at sites of atheroma formation. The modulation of platelet activity using specific pharmacological agents has proven to be a successful strategy for the prevention of thrombosis. In recent years a number of dietary sources of inhibitors of platelet function have been reported (Calzada *et al*. 1997; Cerbone *et al*. 1999; Serebruany *et al*. 2000), although the relationship between diet and platelet function remains unresolved.

Platelets

Platelets circulate in the bloodstream, and play a pivotal role in both health and disease. Their main function is that of haemostasis. The haemostatic mechanisms have a number of important functions: (1) to maintain blood in a fluid state whilst it is in circulation around the body in the vascular system; (2) to arrest bleeding at the site of vascular injury or blood loss by forming a haemostatic plug; (3) to ensure the eventual removal of the haemostatic plug. Platelets are not activated if they are in contact with healthy vascular endothelium due to a combination of control mechanisms exerted by the endothelial cells, such as the synthesis of

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prostacyclin, the capacity to bind thrombin and activate the protein C system, and the ability to inactivate vasoactive substances such as serotonin and bradykinin. When the endothelium is damaged at the site of vascular injury components of the subendothelium, such as collagen, are exposed. Platelets adhere to the subendothelial components and become activated via cell signalling pathways, resulting in shape change, secretion and aggregation. The aggregated platelets form a plug covering the site of damage, which is further consolidated by components of the coagulation system (Hoffbrand & Pettit, 1995).
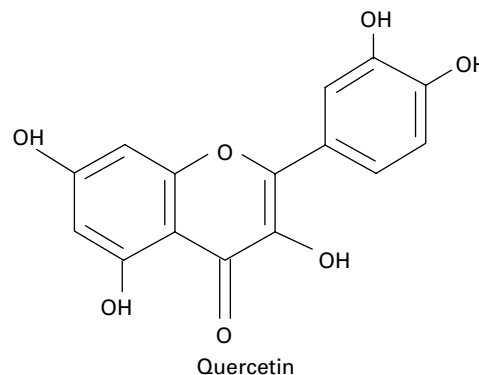
Polyphenolic antioxidants

The polyphenols constitute one of the most numerous and ubiquitous groups of chemicals found in higher plants, and are an integral component of both the human and animal diet. The polyphenols encompass a large number of compounds that range from simple phenolic acids (such as the hydroxycinnamates) to complex polymers (such as the flavonoids and tannins). Plant polyphenols are essential to normal plants. They are involved in plant reproduction and growth, and provide plants with protection against pathogens. The polyphenol content of plants differs between species, and indeed between varieties of the same species (Bravo, 1998; Hollman & Arts, 2000). There are >8000 phenolic structures currently known that are formed as a result of the secondary metabolism of plants. The flavonoids represent the most common and widely distributed group of plant phenolic compounds. Their common structure consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle. Among the classes of flavonoids, those most commonly found are the flavones (e.g. apigenin, luteolin), the flavonols (e.g. quercetin, myricetin, kaempferol) and their glycosides. The flavonoids have been reported to exhibit a wide range of biological effects in animals and man that include antibacterial, antiviral, anti-carcinogenic, anti-inflammatory, anti-allergic anti-thrombotic and antioxidant activity (Bravo, 1998; Vinson, 1998; Hollman & Arts, 2000). Quercetin, the structure of which is shown in Fig. 1, is a major flavonoid and has a wide spectrum of pharmacological effects. Quercetin has been shown to inhibit LDL oxidation *in vitro* (O'Reilly *et al.* 2000) and cause vasodilation in rat aortic strips (Zhao *et al.* 1999).

The dietary intake of the polyphenols, and particularly the flavonoids, has been studied in different countries, and varies greatly around the world. In the Seven Countries Study (Hertog *et al.* 1995) the average intake in west Finland was found to be 2·6 mg/d, whereas in Japan the intake was 70 mg/d. In the UK the intake of flavonols (a subgroup of the flavonoids) has been estimated from the Caerphilly (Wales) study to be 26 mg/d, 82 % of which was provided by tea (Hertog *et al.* 1997).

The flavonoids are present in nearly all plant-derived food and beverage products. For instance, the flavonoid quercetin is found in particularly high levels in apples, onions, wine, tea, kale, broccoli and a number of berries (Hertog *et al.* 1992).

The hydroxycinnamates

The hydroxycinnamic acids are a series of *trans*-phenyl-3-propionoic acids, differing in their ring substitutions. These compounds are widely distributed in plant material, including many foods and beverages. The hydroxycinnamates most commonly found are caffeic acid, ferulic acid, sinapic acid, *p*-coumaric acid and *trans*-cinnamic acid, the structures of which are shown in Fig. 2 (Clifford, 1999; Kroon & Williamson, 1999).

Fig. 1. Chemical structure of the flavonol quercetin. Quercetin has the typical two phenolic ring flavonoid structure and has a number of hydroxyl side chain groups, which confer on the molecule its high antioxidant activity.
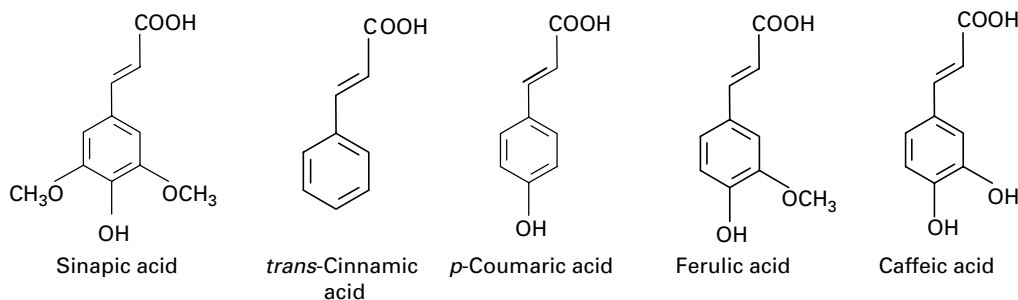
Fig. 2. Chemical structure of the five most common hydroxycinnamates. The hydroxycinnamates all have a single phenolic ring structure and differ in their side chain groups.

Blueberries (*Vaccinium* spp.), aubergines (*Solanum melongena*), wine, grape juice, coffee, apples and citrus juices provide sources of hydroxycinnamates (Clifford, 2000). There are data for dietary intake of hydroxycinnamates from a German study, which investigated the phenolic acid intake of adults in a Bavarian sub-population (Radtke *et al.* 1998). In this study a database of the phenolic content of foods was compiled and used in conjunction with diet diaries to estimate the phenolic acid intake of 119 men and women over a 1-week period. The mean daily intakes of total phenolic acids and total cinnamic acids were 222 and 211 mg respectively, to which caffeic acid (206 mg/d) made the greatest contribution. In this study coffee and fruits were the main sources of hydroxycinnamates.

The bioavailability of polyphenols

The bioavailability of foods relates to the amount of a certain component within ingested food that is able to cross the gut wall and enter the bloodstream, where it may then have a biological effect. However, these components may have been subject to several metabolic transformations within the intestinal mucosa and liver (first pass effects) that may influence their biological potential. Furthermore, the chemical form of polyphenols present in food (e.g. the sugar moiety of flavonoid glycosides) as well as interactions with the food matrix may have profound effects on the bioavailability.

In a study carried out by Conquer *et al.* (1998) twenty-seven men and women were given quercetin-containing capsules supplying 1 g quercetin/d or a placebo for 28 d. Plasma quercetin levels rose from 0·08 (SE 0·08) μM to 1·3 (SE 0·28) μM. In another study (McAnlis *et al.* 1999) five subjects ingested 225 g fried onions (*Allium cepa*), and blood samples were taken after the meal. Plasma quercetin levels were found to peak at 0·77 (SE 0·3) μM 2 h after the meal. Although no detailed information about the bioavailability of quercetin can be derived from these two examples, they demonstrate that quercetin is indeed absorbed and that intake of quercetin-containing foods influences plasma levels of this flavonol. There are also data for the bioavailability of the hydroxycinnamates, including a human dietary study (Bourne & Rice-Evans, 1999) in which four healthy volunteers were supplemented with tomatoes (*Lycopersicon esculentum*; 8/g/kg body weight) providing 21–44 mg ferulic acid. Urine samples were collected for 24 h after consumption and analysed for ferulic acid content. The total free ferulic acid excreted in 8–10 h was 1·9–3·4μM, approximately 4–5 % of the ferulic acid ingested. The polyphenols are very difficult to detect in human and animal tissues, which can be problematic in bioavailability studies.

The role of antioxidants in heart disease

Antioxidants have received attention in recent years as a possible mechanism by which dietary components protect the body from free radicals and reactive oxygen species. These oxidative agents are thought to play a role in many disease states, such as cancer and atherosclerosis (Holvoet & Collen, 1994). Observational studies have shown that individuals who consume large amounts of fruit and vegetables have lower rates of CHD (Hertog *et al.* 1993). This outcome has been attributed to the high levels of antioxidant vitamins found in these foods. One such study is the Oxford Vegetarian study, which was carried out between 1980 and 1993 and included 6115 vegetarian subjects and 5015 meat-eating subjects. The death rate from CHD among the vegetarians was half that of the meat eaters. However, after adjustment for confounding factors, such as BMI, smoking and social class, this difference was no longer significant (Thorogood, 1997).

A number of dietary antioxidants found in fruit and vegetables, such as vitamin E, vitamin C and the polyphenols, have also been shown to be associated with a decreased risk of CHD. The vitamin E intake of >39 000 male health-care professionals was investigated for 4 years (Rimm *et al.* 1996), and those subjects with a dietary intake of vitamin E between 100 and 249 mg/d had a 50 % decreased relative risk of CHD. The effect of vitamin C intake on CHD risk was investigated in the Finnish Mobile Clinic Study (Knekt *et al.* 1994). In this study high vitamin C intake was associated with a reduced risk of CHD mortality in women but not in men. Women with a vitamin C intake of >91 mg/d had a 50 % decreased relative risk of CHD mortality compared with those with a lower intake.

There have been a number of studies in which the relationship between the intake of polyphenols and CHD risk has been investigated. The Zutphen Study carried out in The Netherlands assessed the intake of flavonoids (a subgroup of the polyphenols) of 805 men aged 65–84 years in 1985, with a 5-year follow up. The subjects were grouped according to flavonoid intake (mg/d): high >30, medium 19–29, low <19. The relative risk of both CHD and myocardial infarction was significantly lower in the medium- and high-flavonoid intake groups ($P = 0·015$). These effects were attributed to the flavonoids present in tea, apples and onions, which provide the major sources of flavonoids in the Dutch diet (Hertog *et al.* 1993).

Inhibition of platelet function by antioxidants

In view of the importance of the role of platelets in heart disease, there has been a large amount of research committed to understanding platelet function and the role of platelets in disease. This research has concentrated on finding compounds that can inhibit platelet function and, therefore, decrease the likelihood of thrombosis and thus heart disease. Platelets can be inhibited by a number of dietary components. The dietary antioxidants vitamins C and E (Salonen *et al.* 1991; Freedman *et al.* 1996) and the flavonoids have been shown to have inhibitory effects on platelet function *in vitro* and *in vivo*.

The *in vitro* inhibitory effects of the flavonoids on platelet aggregation have been extensively investigated (Beretz *et al.* 1982; Landolfi *et al.* 1984). Beretz *et al.* (1982) studied thirteen different flavonoids, with thrombin, collagen and ADP as agonists. The most potent inhibitor of platelet aggregation was found to be phloretin (median inhibitory concentration (IC_{50}) 13 μM, with collagen (2·5 μg/ml) as the agonist). Landolfi *et al.* (1984) studied fifteen different flavonoids with arachidonic acid and collagen as agonists. This study reported flavone (IC_{50}

7·2 µM, with arachidonic acid (150 µM) as the agonist) to be the most potent inhibitor of platelet aggregation. The IC$_{50}$ values for quercetin with collagen as the agonist were 55 µM (Beretz *et al.* 1982) and >200 µM (Landolfi *et al.* 1984).

There is also evidence for *in vivo* inhibition of platelet aggregation by flavonoids from dietary sources. Keevil *et al.* (2000) supplemented ten subjects with purple-grape juice, orange juice or grapefruit juice (5·0–7·5 ml/kg per d) for 1 week in a randomised cross-over study. Drinking purple-grape juice for 1 week reduced whole-blood platelet aggregation in response to collagen (1·0 µg/ml) by 77 %. However, consumption of orange juice or grapefruit juice had no effect on platelet aggregation. The purple-grape juice was found to have three times the polyphenolic content of the other two juices, whereas the vitamin C levels were similar for all three juices (Keevil *et al.* 2000). Similarly, Janssen *et al.* (1998) carried out *in vivo* supplementation studies with quercetin, in the form of onions. Eighteen volunteers were fed 220 g onions/d (providing 114 mg quercetin/d) or placebo, each for 7 d, in a randomised crossover experiment. In this study plasma quercetin levels were raised to 1·5 µmol/l. Platelet aggregation for subjects supplemented with onions was inhibited by 7·6 % with collagen (2·0 µg/ml) as agonist and 14 % with ADP (3·0 µM) as agonist, the results were not statistically significant (Janssen *et al.* 1998). While these studies investigated the effects of polyphenolic compounds derived from food on platelet aggregation, Conquer *et al.* (1998) investigated the effects of flavonoid supplementation in the form of capsules. In this study twenty-seven subjects ingested 1 g quercetin and 1 g mixed flavonoids/d or placebo for 28 d. Plasma quercetin concentrations increased from 0·10 µM (SE 0·09) before supplementation to 1·5 µM (SE 0·30) after the 28 d supplementation period. Platelet aggregation was also measured before and after supplementation, with collagen (10 µg/ml) as agonist, and no significant changes in platelet aggregation were observed with quercetin supplementation (Conquer *et al.* 1998).

The present study investigated the effects of five hydroxycinnamates and quercetin on platelet aggregation *in vitro* and examined possible mechanisms for this inhibitory action. In addition, a human dietary study is described in which the effects of a diet rich in fruit and vegetables on platelet aggregation were analysed.

Methods

Materials

Quercetin dihydrate, ferulic acid, *trans*-cinnamic acid, *p*-coumaric acid, sinapic acid and caffeic acid were purchased from Sigma (Poole, Dorset, UK) and solubilised in dimethylsulphoxide, also purchased from Sigma. Horm-Chemie collagen (collagen fibres from equine tendons) was purchased from Nycomed (Munich, Germany). Anti-phosphotyrosine monoclonal antibody (4G10) was obtained from Upstate Biotechnology (TCS Biologicals, Botolph Claydon, Bucks., UK). Horseradish peroxidase conjugated secondary antibodies and the enhanced chemiluminescence detection system were purchased from Amersham Biosciences (Little Chalfont, Bucks., UK). Fura-2 AM was obtained from Molecular Probes (Cambridge Bioscience, Cambridge, UK). ADP was purchased from Chrono-log Corporation (Havertown, PA, USA). All other reagents were obtained from sources previously described (Gibbins *et al.* 1998; Cicmil *et al.* 2000).

Preparation and stimulation of platelets for in vitro studies

Human platelets from healthy aspirin-free volunteers were prepared on the day of the experiment by differential centrifugation as described previously (Gibbins *et al.* 1998) and suspended in modified Tyrodes-Hepes buffer (134 mM NaCl, 0·34 mM Na$_2$HPO$_4$, 2·9 mM KCl, 12 mM NaHCO$_3$, 20 mM Hepes, 5 mM glucose, 1 mM MgCl$_2$, pH 7·3) to a density of 2×10^8 cells/ml for aggregation experiments. Stimulation of platelets (450 µl) with collagen (50 µl) was performed at 37° using an optical platelet aggregometer (Chrono-log Corporation) with continuous stirring (1200 rpm; Born, 1962). Platelets were incubated with polyphenol dissolved in dimethylsulphoxide (1 µl) or with dimethylsulphoxide alone (0·2 % (v/v); 1 µl) for 5 min (after stirring for 10 s) followed by stimulation with collagen for 90 s. For immunoblotting experiments, platelets were suspended at 8×10^8 cells/ml in buffer containing 1 mM-ethylene glycol-O,-O'-bis(2-amino-ethyl)-N,N,N',N'-tetra-acetic acid to prevent aggregation. Hence, the concentration of collagen was increased to 25 µg/ml for the observation of tyrosine phosphorylation events. Polyphenol concentrations were therefore increased proportionately. Platelet stimulation was terminated by the addition of an equal volume of ice-cold NP40 lysis buffer (Nonidet P40; 20 mM-Tris, 300 mM-NaCl, 10 mM-EDTA, 1 mM-phenylmethylsulfonyl fluoride, 2 mM-Na$_3$VO$_4$, 10 µg/ml leupeptin, 10 µg/ml aprotonin, 1 µg/ml pepstatin A, pH 7·3; 2%, v/v). Detergent-insoluble debris was removed by centrifugation and proteins were separated by SDS–PAGE under reducing conditions using 10 % (v/v) gels and transferred to polyvinylidene difluoride membranes by semi-dry Western blotting.

Immunoblotting

Membranes were blocked by incubation in bovine serum albumin (10 %, w/v) dissolved in Tris-buffered saline–Tween (20 mM-Tris, 137 mM-NaCl, Tween 20 (0·1 %, v/v), pH 7·6). Primary and secondary antibodies were diluted in Tris-buffered saline–Tween containing bovine serum albumin (2 %, w/v) and incubated with membranes for 1 h at room temperature with rotation. Blots were washed for 2 h in Tris-buffered saline–Tween following each of the incubations with antibodies and then developed using an enhanced chemiluminescence detection system. Anti-phosphotyrosine antibody (4G10) was used at a concentration of 1 µg/ml and horseradish peroxidase-conjugated secondary antibody (anti-mouse-IgG-horseradish peroxidase) was diluted 1:10 000. Densitometry analysis was carried out using a Bio-Rad GS710 densitometer with Quantity One analysis software (Bio-Rad, Hemel Hempstead, Herts., UK).

Measurement of intracellular calcium ion concentration by spectrofluorimetry

Washed human platelets (as described earlier) were incubated at 2×10^9 cells/ml in Ca^{2+}-free Tyrodes-Hepes buffer with $3\,\mu M$-Fura-2 AM for 45 min. Platelets were washed once and resuspended at 2×10^8 cells/ml in modified Tyrodes-Hepes buffer. Platelets were incubated with quercetin or dimethylsulphoxide alone (as described earlier) for 5 min and then stimulated with collagen ($5 \cdot 0\,\mu g/ml$) with constant stirring at $37°$ in a luminescence spectrophotometer (LS-50B, Perkin Elmer, Beaconsfield, Bucks., UK) with excitation wavelengths of 340 nm and 380 nm. Fluorescence emission was measured at a wavelength of 510 nm. The ratio of emission values (340 nm:380 nm) was calculated and converted to Ca^{2+} concentration using FLWinLab software (Perkin Elmer) and the equation:

$$[Ca^{2+}]_i = (K_d \times (R - R_{min})) \div ((R_{max} - R) \times SFB),$$

where $[Ca^{2+}]_i$ is the intracellular Ca^{2+} concentration, R is the value for the emission ratio (340 nm:380 nm), K_d is the dissociation constant of the $Fura-2 - Ca^{2+}$ complex (224 nM) and SFB is the fluorescence ratio at 340 nm:380 nm of R_{min} and R_{max}. R_{max}, the maximum 340 nm:380 nm value, was determined by lysing platelets with NP40 immunoprecipitation lysis buffer in the presence of $1\,mM$-$CaCl_2$. R_{min}, the minimum 340:380 nm value, was determined by the addition of $2\,mM$-ethylene glycol-O,-O'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid.

Design of fruit- and vegetable-rich diet study

Twenty subjects (ten male and ten female) were recruited from the University of Reading. The Ethics and Research Committee of the University of Reading approved the protocol and all subjects gave informed consent before taking part. For 3 d the subjects followed a fruit- and vegetable-free diet. A blood sample was taken at the end of the 3 d, and the subjects were given a lunchtime meal of five portions of fruit and vegetables (Table 1) every day for the next 5 d. At the end of this period another blood sample was taken. All subjects were asked to abstain from polyphenol-rich foods for the duration of the study, so that the only polyphenols in the diet were those in the fruit and vegetable meal. The subjects were given details of a diet low in polyphenols (Hertog et al. 1992), and they were asked to record all food and drink consumed throughout the 8 d of the study. A maximum of three cups of tea or coffee per d was allowed and the amount consumed was noted in the diet diary.

The US Department of Agriculture (1992) Food Pyramid Guide suggests five or more servings of fresh fruit and vegetables per d. This recommendation was used as a guide for the design of a meal that contained five or more servings of fruit and vegetables that were high in polyphenols and also provided carbohydrate and protein. The composition of the meal is shown in Table. 1.

Measurement of whole-blood platelet aggregation in samples from the fruit and vegetable study

Blood samples were taken into $4 \cdot 5$ ml vacutainer system blood tubes (Becton Dickinson, Plymouth, UK) containing

Table 1. Composition of fruit and vegetable meal given to subjects participating in the fruit- and vegetable-rich diet study*

Course	Food product	Weight (g)
Salad starter	Cherry tomatoes	100
	Cucumber	60
	Continental four-leaf salad	25
	Salad dressing	4
Main course	Tomato and onion pasta sauce composed of:	175
	Yellow onions	approximately 70
	Passata (sieved plum tomatoes)	approximately 105
	Extra virgin olive oil	>1
	Tricolour fusilli pasta (cooked weight)	75 or 125 †
Dessert	Frozen raspberries (defrosted weight)	100
	Vanilla ice cream	25
	One apple	average 30
Drink	Purple grape juice	350 ml

*For details of procedures, see p. 472.
†Subjects weighing <60 kg were given 75 g pasta, those weighing >60 kg were given 125 g, unless otherwise requested.

$0 \cdot 105$ M-sodium citrate as anticoagulant (1 vol. sodium citrate:9 vol. blood). Samples were stored at room temperature, and analysis was commmenced 1 h after venepuncture and completed within 4 h.

Whole blood was diluted 1:1 (v/v) with PBS, followed by pre-incubation to $37°$. Whole-blood platelet aggregation was measured for 6 min after stimulation with an agonist. The extent of aggregation was determined as the amplitude of the slope produced after the addition of the agonist (electrical impedance; Ω).

Plasma concentrations of flavonoids

The plasma concentrations of flavonoids were determined as described previously (Ader et al. 2000), with minor modifications. Blood samples were taken into 10 ml vacutainer system blood tubes with EDTA. Plasma was prepared by centrifugation for 10 min at $2000\,g$, and stored at $-80°$ until analysed. An aliquot (800 µl) of the plasma sample was acidified with 100 µl acetic acid ($0 \cdot 583$ mol/l), spiked with 20 µl rhamnetin (50 µg/ml in methanol; internal standard) and subsequently treated with a mixture of β-glucuronidase (10^4 units)–sulphatase ($2 \cdot 5 \times 10^2$ units); crude extract from *Helix pomatia*; Sigma-Aldrich AG, Deisenhofen, Germany. After an incubation of 1 h at $37°$ the flavonol aglyca quercetin, kaempferol, isorhamnetin and tamarixetin were simultaneously extracted with $5 \cdot 5$ ml acetone. The mixture was centrifuged at $3700\,g$ for 45 min and the supernatant fraction was evaporated to dryness under vacuum at $45°$ (SpeedVac AES 1010; Savant Instruments Inc., Farmindale, NY, USA). The residue was redissolved in 200 µl methanol, $77 \cdot 5$ µl deionised water and $22 \cdot 5$ µl HCl (10 mol/l). For HPLC analysis 30 µl were injected onto a C-18 Kromasil 100 column (250×4 mm, particle size 5 µm; Jasco, Gross-Umstadt, Germany) protected by a C-18 Inertsil ODS-2 precolumn (10×4 mm, 5 µm particle size; Jasco). The eluent (1 ml/min) was $0 \cdot 025$ M-NaH_2PO_4 (pH $2 \cdot 4$)–acetonitrile–

methanol (68:27:5, by vol.). The column effluent was mixed with 1·5 M-Al(NO$_3$)$_3$ in methanol containing acetic acid (7·5 %, v/v) at a rate of 0·4 ml/min in a post-column reactor(3 m Tefzel-tube, 0·5 mm i.d., coiled to an outer diameter of 5 mm and connected to the HPLC column with a low-dead-volume tee; Jasco). The column and the reactor were placed in a column oven set at 30°. The fluorescence of the resulting flavonoid–metal complex was measured at 485 nm using a Jasco FP 920 (Jasco Corp) fluorescence detector with excitation wavelength set at 422 nm. The limit of detection was 3 ng/ml.

Statistical analysis

IC$_{50}$ values were calculated using Prism for Windows version 2.0 (Graphpad Inc., San Diego, CA, USA) with non-linear regression. Graphs were plotted using a sigmoidal curve, and variable Hill slope with 100% and 0% inhibition constants. Paired *t* tests were performed using SPSS 10.0 for windows (SPSS, Chicago, IL, USA). Results are presented as mean values with their standard errors and $P \leq 0.05$ was considered significant.

Results

In vitro effects of polyphenols on platelet function

The effects of five different hydroxycinnamates and quercetin on platelet aggregation were investigated. Percentage inhibition of platelet aggregation was calculated and IC$_{50}$ values determined. The IC$_{50}$ values for the hydroxycinnamates are shown in Table 2. Sinapic acid had the highest IC$_{50}$ value (815 (SE 1·09) μM); all the other IC$_{50}$ values were approximately 500 μM.

The IC$_{50}$ values for quercetin with collagen (4, 2 and 0·5 μg/ml) as agonist were (μM) 6·47 (SE 1·09), 4·87 (SE 1·11) and 2·37 (SE 1·03) respectively (*n* 3). The IC$_{50}$ value for quercetin with thrombin (0·1 IU/ml) as agonist was 27·6 (SE 1·04) μM (*n* 3). The IC$_{50}$ values for quercetin were all substantially lower than those for the hydroxycinnamates. The IC$_{50}$ values were also dependent on the concentration of collagen used.

The effect of quercetin on the mobilisation of Ca from intracellular stores was investigated. Mean peak Ca concentrations are shown in Table 3. When platelets were incubated with varying concentrations of quercetin, mean peak Ca

Table 2. Median inhibitory concentration (IC$_{50}$) values for the inhibition of platelet aggregation by five hydroxycinnamates with collagen (1·0 μg/ml) as agonist*

(Mean values with their standard errors for three determinations)

Hydroxycinnamate	IC$_{50}$ (μM)	
	Mean	SE
Sinapic acid	815·9	1·09
trans-Cinnamic acid	528·5	1·19
p-Coumaric acid	482·6	1·22
Ferulic acid	481·7	1·20
Caffeic acid	478·1	1·19

*For details of procedures, see p. 472.

Table 3. Inhibition of platelet calcium release by quercetin with collagen (5·0 μg/ml) as agonist.

(Mean values with their standard errors for three determinations)

| Quercetin (μM)	Peak Ca concentration (nM)	
	Mean	SE
0	87·75	7·76
5	75·84	13·7
10	51·98	15·7
15	21·73	4·96
20	3·7	2·51

*For details of procedures, see p. 472.
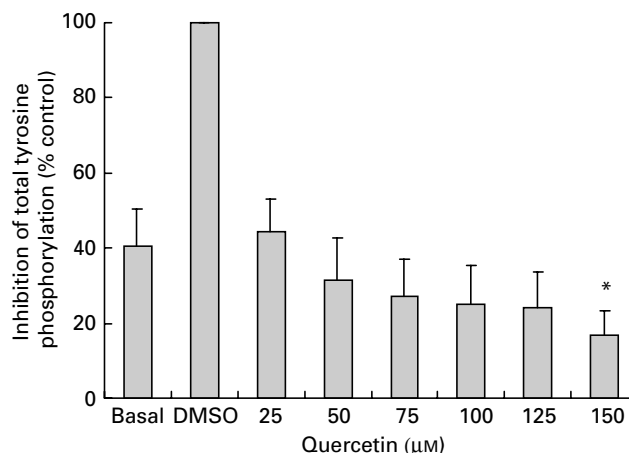
Fig. 3. Quercetin inhibits collagen-stimulated whole-cell protein-tyrosine phosphorylation. Platelets, in the presence of ethylene glycol-*O*,-*O'*-bis(2-amino-ethyl)-*N*,*N*,*N'*,*N'*-tetraacetic acid (1 mM), were incubated with quercetin (25–150 μM) or dimethylsulphoxide (DMSO) control for 5 min and then stimulated with collagen (25 μg/ml) for 90 s. Platelets were then lysed and proteins separated by SDS–PAGE under reducing conditions and subjected to immunoblotting to detect protein phosphotyrosine residues. Total protein-tyrosine phosphorylation was analysed using densitometry. Total tyrosine phosphorylation is shown as a percentage of the control value. Values are means with their standard errors represented by vertical bars for three determinations. Mean value was significantly different from that for the dimethylsulphoxide control and from basal levels: *$P = 0.05$.

release was inhibited in a concentration-dependent manner, and was almost completely abolished by 20 μM-quercetin.

The effect of quercetin on platelet whole-cell protein tyrosine phosphorylation was also investigated. Fig. 3 shows the total platelet whole-cell tyrosine protein phosphorylation as a percentage of the control value. Percentage total platelet whole-cell tyrosine phosphorylation increased from basal levels on stimulation with collagen, as previously reported (Gibbins *et al.* 1997). In the presence of quercetin, percentage total platelet whole-cell protein tyrosine phosphorylation was inhibited in a concentration-dependent manner. Quercetin at 150 μM significantly inhibited percentage total platelet whole-cell tyrosine phosphorylation when compared with the dimethylsulphoxide control and basal levels ($P = 0.05$).
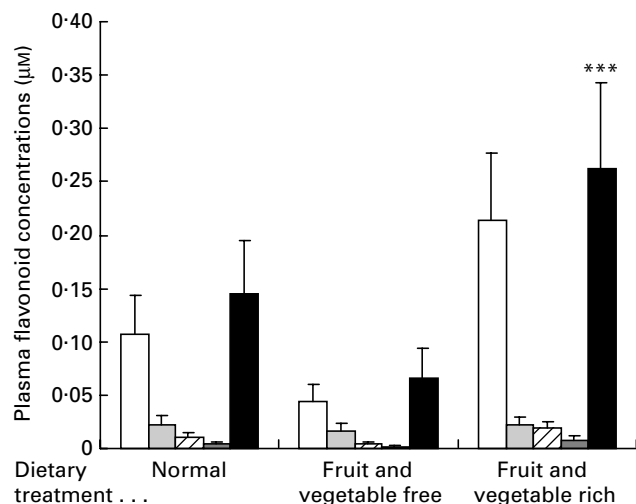
Fig. 4. The effect of polyphenolic content of the diet on plasma flavonol levels. The concentrations of four flavonols (quercetin (▢), kaempferol (▨), isorhamnetin (▨) and tamarixetin (▨)) in blood plasma from subjects consuming their normal diet, after 3 d on a low-polyphenol diet and after 5 d of supplementation of the low-polyphenol diet with a fruit- and vegetable-rich lunchtime meal every day were measured by HPLC (for details of diets and procedures, see p. 472). Total flavonol concentration (■) represents the sum of the concentrations of the four flavonols measured. Values are means with their standard errors represented by vertical bars for twenty subjects. Mean value was significantly different from that after the 3 d period on the fruit- and vegetable-free diet: ***$P = 0.001$

The effects of a fruit and vegetable-rich diet on platelet aggregation

Plasma concentrations of quercetin, kaempferol, isorhamnetin and tamarixetin were measured as markers of polyphenolic intake. The mean plasma concentrations of these polyphenols are shown in Fig. 4. Plasma concentrations of the four flavonol compounds were measured on entry to the study (i.e. under normal dietary conditions), after the fruit- and vegetable-free period and after the fruit- and vegetable-enriched period. The total plasma flavonol concentration showed a decrease after the 3 d fruit- and vegetable-free period compared with that for the normal diet (from 0.154 μM (SE 0.027) to 0.071 μM (SE 0.015)), but increased significantly (from 0.071 μM (SE 0.015) to 0.277 μM (SE 0.044); $P = 0.001$) after supplementation with the fruit- and vegetable-rich lunchtime meal for 5 d. Quercetin concentrations were the highest of the four compounds detected and constituted 80 % of the total levels.

Platelet aggregation was measured as whole-blood platelet aggregation with two different agonists (ADP and collagen) after the fruit- and vegetable-free period and after the fruit- and vegetable-supplementation period for the twenty subjects. After the supplementation period the mean whole-blood platelet aggregation had decreased from 13.56 (SE 0.54) Ω to 12.32 (SE 0.78) Ω with ADP (10 μM) and from 13.35 (SE 1.60) Ω to 12.9 (SE 1.57) Ω with collagen (1.5 μg/ml). However, these changes were not significant.

When a higher dose of collagen (5.0 μg/ml) was used as the agonist the mean whole-blood platelet aggregation after the supplementation period was unchanged.

Discussion

The dietary antioxidants found in fruits and vegetables have received attention in recent years as a possible mechanism by which dietary components protect the body from free radicals and reactive oxygen species. These dietary antioxidants may therefore play a role in many disease states, such as atherosclerosis. Platelet function can be regulated by a number of pharmacological agents, including a number of compounds that are found in the diet (Cerbone *et al.* 1999; Serebruany *et al.* 2000). A group of polyphenolic antioxidants of the flavonoid family have been shown to inhibit platelet aggregation *in vitro* (Beretz *et al.* 1982; Landolfi *et al.* 1984; Tzeng *et al.* 1991) and some human dietary studies have also suggested that a diet high in flavonoids may inhibit platelet aggregation *in vivo* (Janssen *et al.* 1998; Keevil *et al.* 2000). Quercetin, a flavonoid that has received much attention in the area of nutritional biology, has been shown to inhibit platelet aggregation *in vitro* (Beretz *et al.* 1982). Although a specific mechanism for this inhibitory activity has not been established, a number of investigations of possible mechanisms have been reported. Certain flavonoids have been shown to inhibit the generation of metabolites of arachidonic acid by cyclooxygenase and lipoxygenase (Beretz *et al.* 1982; You *et al.* 1999), and inhibit Ca-dependent isoforms of protein kinase C (Liu & Liang 2000). However, platelet function is controlled by a network of signalling pathways, and the exact mode of action has not been elucidated.

In the present study the effects of the hydroxycinnamates (a subgroup of the polyphenols) and the flavonol quercetin on platelet aggregation *in vitro* were investigated. The hydroxycinnamates were found to inhibit platelet aggregation with collagen as an agonist. However, the concentrations of hydroxycinnamates required (556 μM is the average concentration required to produce a 50% inhibition of platelet aggregation) were higher than those reported to be present in human plasma (approximately 3 μM; Bourne & Rice-Evans, 1998, 1999). Thus, the hydroxycinnamates would not appear to make a marked contribution to reduced platelet function *in vivo*.

Consistent with previous reports (Beretz *et al.* 1982), quercetin was shown to inhibit collagen-induced platelet aggregation *in vitro* in a concentration-dependent manner. This effect was also dependent on the concentration of collagen used, with higher concentrations of quercetin being required to overcome stimulation by higher concentrations of collagen. The IC_{50} values for quercetin found in the present study are lower than those published previously (Beretz *et al.* 1982; Landolfi *et al.* 1984), and are the same, or close to, quercetin concentrations that may be present in the human plasma, which can be as high as 3.5 μM after ingestion of certain forms of quercetin (Hollman *et al.* 1999). Thus, quercetin derived from the normal diet or through supplementation may have an inhibitory effect on platelet function *in vivo*.

In the present study quercetin was also shown to inhibit thrombin-induced platelet aggregation. However, as quercetin has a more potent inhibitory effect on collagen-stimulated platelet aggregation than on thrombin-induced platelet aggregation, the effect of quercetin on the collagen-stimulated signalling pathway was examined further. Quercetin was found to inhibit both collagen-stimulated Ca^{2+} mobilisation from intracellular stores and collagen-stimulated whole-cell protein-tyrosine phosphorylation in a concentration-dependent manner. These processes are both essential for platelet activation and play a major role in platelet aggregation (Smith *et al.* 1992; Poole *et al.* 1997). Further investigation of the specific proteins involved in the collagen signalling pathway via the collagen receptor glycoprotein VI are required to further elucidate the role of quercetin as an inhibitor of platelet function.

The effect of a polyphenol-rich diet on platelet function was investigated by providing twenty subjects on a polyphenol-restricted diet with a lunchtime meal consisting of five portions of fruit and vegetables. The lunchtime meal was designed to contain a number of different sources of foods that were high in certain polyphenols; the levels of polyphenols in the meal are currently under investigation.

The levels of polyphenolic compounds present in the blood plasma were measured using four markers of polyphenol intake, quercetin, kaempferol, isorhamnetin and tamarixetin. In many foods quercetin is the most abundant of the flavonols and is therefore an appropriate marker of polyphenol intake. Flavonol concentrations were measured before entry into the study, as a measure of polyphenol intake under normal dietary conditions. The total plasma flavonol concentration was 0·154 μM and the quercetin concentration was 0·1 μM. The total plasma concentration of the four flavonols and that of quercetin decreased by 50 % after 3 d on the fruit- and vegetable-free diet. This effect, which has not previously been reported, could be used as a measure of compliance with the fruit- and vegetable-free diet. After the 5 d fruit- and vegetable-supplementation period there was a significant increase in the total plasma flavonol ($P = 0·001$) and quercetin concentrations. The total plasma flavonol concentration was twice that observed with the normal diet, suggesting that supplementation of the diet with polyphenols had been effective. The plasma concentration of quercetin was, however, relatively low. Concentrations of 1·5 μM (SE 0·39) after a 7 d period of supplementation with onion soup containing 114 mg quercetin (Janssen *et al.* 1998) and 1·34 μM after supplementation of subjects with onions containing 134 mg quercetin (Aziz *et al.* 1998) have been reported, while a higher plasma quercetin concentration (3·5 μM) was found after subjects ingested 144 mg pure quercetin-4′-*O*-β-D-glucoside in the form of a drink (Hollman *et al.* 1999). The present study was designed to supplement the diet with a number of different polyphenolic compounds, in addition to the flavonoids. Thus, a measure of the total polyphenol content of the plasma samples is required.

Whole-blood platelet aggregation measured after the 3 d fruit- and vegetable-free period and after the 5 d fruit- and vegetable-supplementation period indicated no significant inhibition. However the plasma quercetin concentration after the fruit- and vegetable-supplementation period were lower than those previously reported values, whereas the levels required for the inhibition of platelet aggregation *in vitro* were several-fold higher. If plasma quercetin concentration had been within the range reported by Hollman *et al.* (1999; 3·5 μM) a significant reduction in platelet aggregation may have been observed. Since inhibition of platelet aggregation by quercetin has been observed with lower concentrations of agonist, a lower concentration of either ADP or collagen may have yielded clearer differences in platelet aggregation after the period of fruit and vegetable supplementation. Furthermore, the plasma total polyphenol levels could be several times higher than those of quercetin, and it has been suggested that a number of polyphenolic compounds are able to inhibit platelet function *in vitro* (Beretz *et al.* 1982; Landolfi *et al.* 1984), although such activity was not apparent in the current study.

Although previous studies of the effects of a diet rich in polyphenols have reported inconsistent results (Conquer *et al.* 1998; Keevil *et al.* 2000), they do suggest that certain types of food that are richer in polyphenols may inhibit platelet function. Keevil *et al.* (2000) used purple-grape juice, a very potent inhibitor of platelet function, as a supplement, whereas Janssen *et al.* (1998) used onions and reported less potent effects. In the present study a mixture of different foods that included both onions and purple-grape juice was used, but no significant effects on platelet aggregation were found. Thus, further investigation of the polyphenolic compounds present in foods that are able to inhibit platelet function, such as those found in purple-grape juice and red wine (Russo *et al.* 2001), is required. Grape-derived products contain a variety of polyphenolic compounds such as tannins and anthocyanins, which may also be able to inhibit platelet function. Furthermore, the polyphenolic compounds found in drinks such as wine, tea and fruit juice may be more bioavailable (Hollman *et al.* 1997) than those in solid food matrices. This factor may contribute to the more potent inhibitory effects on platelet function seen with these foods.

It has been shown that certain polyphenolic compounds are able to inhibit platelet aggregation *in vitro* and that some of these compounds are more effective inhibitors than others. Whether this difference in potency is a function of structure or antioxidant activity is unknown. The precise mechanism of action of quercetin in relation to platelet function is currently under investigation. Although supplementation of the diet with foods high in certain polyphenols has been shown to inhibit platelet function *in vivo*, the effect of a diet rich in a variety of polyphenols has yet to be resolved. Investigation of the effects of individual polyphenols and combinations of these compounds on platelet function may explain the different outcomes that have been reported. The role of dietary polyphenolic compounds as inhibitors of platelet function remains to be fully elucidated.

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