Ingestion of onion soup high in quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in man: a pilot study

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Epidemiological data suggest that those who consume a diet rich in quercetin-containing foods may have a reduced risk of CVD. Furthermore, *in vitro* and *ex vivo* studies have observed the inhibition of collagen-induced platelet activation by quercetin. The aim of the present study was to investigate the possible inhibitory effects of quercetin ingestion from a dietary source on collagen-stimulated platelet aggregation and signalling. A double-blind randomised cross-over pilot study was undertaken. Subjects ingested a soup containing either a high or a low amount of quercetin. Plasma quercetin concentrations and platelet aggregation and signalling were assessed after soup ingestion. The high-quercetin soup contained 69 mg total quercetin compared with the low-quercetin soup containing 5 mg total quercetin. Plasma quercetin concentrations were significantly higher after high-quercetin soup ingestion than after low-quercetin soup ingestion and peaked at 2·59 (SEM 0·42) μmol/l. Collagen-stimulated (0·5 μg/ml) platelet aggregation was inhibited after ingestion of the high-quercetin soup in a time-dependent manner. Collagen-stimulated tyrosine phosphorylation of a key component of the collagen-signalling pathway via glycoprotein VI, Syk, was significantly inhibited by ingestion of the high-quercetin soup. The inhibition of Syk tyrosine phosphorylation was correlated with the area under the curve for the high-quercetin plasma profile. In conclusion, the ingestion of quercetin from a dietary source of onion soup could inhibit some aspects of collagen-stimulated platelet aggregation and signalling *ex vivo*. This further substantiates the epidemiological data suggesting that those who preferentially consume high amounts of quercetin-containing foods have a reduced risk of thrombosis and potential CVD risk.

Quercetin: Platelets: glycoprotein VI: Phytochemicals: CVD: Signalling

The relationship between a diet high in fruits and vegetables and a decreased risk of CVD is well established, although an explanation for this is far from clear. The relationship between diet and platelet function is complex and is not understood. A number of studies have focused on the effects of dietary antioxidants on platelet function, among which are the flavonols. The flavonols are a subgroup of polyphenolic compounds present in fruits and vegetables and are thought to play a role in the reduction of incidence of chronic diseases (Hertog *et al.*1995, 1997); they have also been reported to inhibit platelet aggregation (Beretz *et al.* 1982; Landolfi *et al.* 1984; Hubbard *et al.* 2003, 2004). Quercetin (and its conjugate forms) is a key flavonol in the human diet and is found in high levels in onions, apples, tea and wine (Hertog *et al.* 1992). One of the quercetin conjugates, quercetin 4′-glucoside, is a principal form of quercetin in the diet, particularly high levels being present in onions. This form has been found to be preferentially absorbed in the gut in human studies involving single meals or supplementation with purified compound

(Aziz *et al.* 1998; Hollman *et al.* 1999; McAnlis *et al.* 1999; Hubbard *et al.* 2004). Quercetin has been shown to be a very potent bioactive molecule by possessing anti-inflammatory, anti-allergenic, antiviral, anticancer, antioxidant and anti-thrombotic actions, among others (reviewed by Formica & Regelson, 1995).

We have recently reported that quercetin and quercetin-4′-glucoside potently inhibit collagen-stimulated platelet aggregation and collagen receptor (glycoprotein VI (GPVI))-mediated signalling *in vitro* and *ex vivo* (Hubbard *et al.* 2003, 2004). Quercetin has been shown to preferentially inhibit collagen-stimulated platelet aggregation and the tyrosine phosphorylation and/or activity of a number of critical components of the GPVI signalling pathway *in vitro*. These include the non-receptor tyrosine kinase Syk, phospholipase Cγ2 (PLCγ2) and phosphoinositide 3-kinase. The inhibition concentration$_{50}$ values for the inhibition of platelet aggregation by quercetin were below 3 μmol/l when platelets were stimulated with concentrations of collagen between 0·5 and 1·0 μg/ml

(Hubbard *et al.* 2003). The *in vivo* effects of quercetin on platelet function were demonstrated after subjects ingested purified quercetin 4′-glucoside. Plasma quercetin levels peaked at 9·7 μmol/l, and collagen-stimulated platelet aggregation and signalling via GPVI were significantly inhibited after the ingestion of both 150 and 300 mg doses of quercetin 4′-glucoside (Hubbard *et al.* 2004). The effects of supplementation with quercetin 4′-glucoside on platelet signalling and function mirrored those observed in the *in vitro* study.

The anti-thrombotic effects of quercetin derived from a dietary source have not been investigated. In the present study, we have investigated the possible inhibitory effects of quercetin ingestion from a dietary source on collagen-stimulated platelet aggregation and signalling.

Subjects and methods

Human supplementation study design

Consent from the University of Reading Ethics Committee was obtained before the start of the study. Two types of onion soup (see later for details), one high in quercetin glucosides and the other with low quercetin glucoside levels, were ingested to investigate the effects of quercetin on collagen-stimulated platelet function and signalling. The study was a two-treatment, randomised, double-blind, cross-over study in which six subjects (four men and two women; age 34 (SD 7) years; weight 68·5 (SD 3·6) kg; BMI 23·1 (SD 2·0) kg/m^2) abstained from ingesting aspirin and complied with a low-quercetin diet for 14 d prior to the study to ensure quercetin washout (foods low in quercetin were defined according to Hertog *et al.* (1992)). Following a 12 h overnight fast, the subjects then took part in a 1 d study. They were cannulated, and a baseline blood sample was taken. The subjects then ingested either the high- or the low-quercetin soup (600 ml), the order being randomised. Blood samples were then taken at 30, 60, 90, 120, 150, 180, 210, 240, 300, 360, 390 and 420 min after soup ingestion and at 24 and 32 h for the determination of plasma quercetin level. Blood samples taken at 0, 1 and 3 h were also used to examine the effect of soup ingestion on platelet function and signalling. The study day was followed by a 1-month washout period, after which the subject ingested the remaining treatment.

Soup preparation

Batches of near-isogenic high- and low-quercetin onions (Femia *et al.* 2003) were used to prepare high- and low-quercetin onion soups. The soups were prepared in the same manner on the same day in one batch and then frozen at −20°C. For each person, chopped onions (500 g, equivalent to approx. three medium-sized onions) were added to 500 ml boiling water and blanched for 2 min. The onions were then drained (retaining the blanching liquid) and added to a large saucepan containing 25 g butter and 7·5 ml refined olive oil before being fried for 10 min until they started to brown. Meanwhile, 1·5 rounded teaspoons of Swiss Vegetable Bouillon Powder (Marigold Health Foods, London, UK) was added to the blanching liquid to make a stock, and this was then added to the fried onions. The saucepan was then covered and left to simmer for 30 min. The soup was left to cool and then liquidised; 600 ml portions were then frozen at

−20°C until use. The two soups were analysed for flavonoid content by preparing both non-hydrolysed and hydrolysed extracts (with 0·5 % t-butylated hydroxyquinone as an antioxidant) followed by HPLC, as previously described (Bovy *et al.* 2002). Quercetin was used as a standard to quantify both glycosides and the aglycone (Table 1). On the day of use, the portions of soup were defrosted and then reheated in a microwave. The subjects then consumed the soup within 15 min and were not allowed to ingest any other food or drink, apart from water, during the following 4 h.

Materials

β-Glucuronidase/sulphatase (crude extract from *Helix pomatia*) and protein A-sepharose 4BL were purchased from Sigma (Poole, Dorset, UK). Horm-Chemie collagen (collagen-fibres from equine tendons) was purchased from Nycomed (Munich, Germany). Anti-phosphotyrosine monoclonal antibody (4G10) came from Upstate Biotechnology (TCS Biologicals, Botolph Claydon, Buckinghamshire, UK). Anti-Syk (N-19), anti-Syk (LR) and anti-PLCγ2 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Autogen Bioclear UK Ltd, Calne, Wiltshire, UK). Horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG, anti-mouse IgG), the enhanced chemiluminescence immuno-detection system and Hyperfilm were purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Quercetin, kaempferol, isorhamnetin, tamarixetin, rhamnetin and all chemicals used for preparing the HPLC eluent were purchased from Roth (Karlsruhe, Germany).

Preparation and stimulation of platelets

Platelets were prepared from the blood samples collected from subjects at the 0, 1 and 3 h time points on the day of the experiment by differential centrifugation as previously described (Gibbins *et al.* 1997) and were suspended in modified Tyrodes-Hepes buffer (134 mmol/l NaCl, 0·34 mmol/l Na$_2$HPO$_4$, 2·9 mmol/l KCL, 12 mmol/l NaHCO$_3$, 20 mmol/l Hepes, 5 mmol/l glucose, 1 mmol/l MgCl$_2$, pH 7·3) at a density of 2×10^8 cells/ml for aggregation experiments. Stimulation of platelets (450 μl) with collagen (50 μl; 0·5, 1, 2 and 3 μg/ml final concentration) was performed at 37°C in an optical platelet aggregometer (Chrono-log Corporation, Havertown, PA, USA) with continuous stirring (1200 rpm).

For immunoprecipitation and immunoblotting experiments, platelets were suspended at 8×10^8 cells/ml in Tyrodes-Hepes buffer containing 1 mmol/l EGTA to prevent aggregation.

Table 1. Levels of quercetin in onion soups as ingested (mg/l)

(Values were means of two replicate analyses)

Onion type	Quercetin aglycone	Quercetin 4′-glucoside	Quercetin 3, 4′-glucoside	Total
Low-quercetin onions	0·1	3·8	4·3	8·2
High-quercetin onions	1·1	53·2	60·5	114·8

For details of subjects and procedures, see this page.

Based on previous platelet signalling studies (Hubbard *et al.* 2003, 2004), the concentration of collagen was increased to 25 µg/ml to enable the detection of tyrosine phosphorylation events. This was necessary because, for successful immuno-precipitation of proteins from platelets, they were stimulated in the presence of EGTA to prevent fibrinogen-dependent aggregation. Although this ensured equivalent levels of protein isolation from activated and non-activated platelets, it resulted in lower levels of positive-feedback signalling through the fibrinogen receptor, integrin αIIbβ3, and therefore in comparison to stimulation under aggregating conditions, higher agonist concentrations were required in order to measure protein tyrosine phosphorylation (Cicmil *et al.* 2002).

Platelet stimulation was terminated by the addition of an equal volume of ice-cold NP40 lysis buffer, (2 % v/v Nonidet P40, 20 mmol/l Tris, 300 mmol/l NaCl, 10 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride, 2 mmol/l Na_3VO_4, 10 µg/ml leupeptin, 10 µg/ml aprotonin, 1 µg/ml pepstatin A, pH 7·3). Detergent-insoluble debris was removed by centrifugation (14 000 g for 10 min), and samples were frozen at $-8°C$ until analysis. For immunoprecipitation studies, whole cell lysates were precleared by mixing with protein A-sepharose (60 µl 50 % w/v suspension in Tris-buffered saline-Tween (20 mnol/l Tris, 137 mmol/l NaCl, 0·1 % v/v Tween 20, pH 7·6)) for 1 h at 4°C. Protein A-sepharose was removed by centrifugation before adding the relevant antibody (anti-Syk (LR) or anti-PLCγ2 (1 µg/ml)). Following rotation for 1 h at 4°C, 60 µl protein A-sepharose suspension was added to each sample and rotated for a further 1 h at 4°C, before washing the sepharose pellet and adding the reducing Laemmli buffer. Immunoprecipitated proteins were separated by SDS-PAGE under reducing conditions using 10 % gels and transferred to polyvinylidene difluoride membranes by semi-dry western blotting

Immunoblotting

Membranes were blocked by incubation in 10 % (w/v) bovine serum albumin dissolved in Tris-buffered saline-Tween. Primary and secondary antibodies were diluted in Tris-buffered saline-Tween containing 2 % (w/v) bovine serum albumin, 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 incubation with antibodies and were developed using an enhanced chemiluminescence detection system. Primary antibodies were used at a concentration of 1 µg/ml anti-phosphotyrosine antibody (4G10), anti-Syk (N-19), anti-PLCγ2) and horseradish peroxidase-conjugated secondary antibody (anti-mouse-horseradish peroxidase, anti-rabbit-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, Hertfordshire, UK).

Plasma concentrations of quercetin, isorhamnetin and tamarixetin

Plasma concentrations of flavonoids were measured as previously described (Ader *et al.* 2000). Briefly, blood was taken into 10 ml EDTA vacutainer system blood tubes. Plasma was prepared by centrifugation for 10 min at 2000 g and stored at $-80°C$ until analysis. An 800 µl aliquot of the plasma sample was acidified with 100 µl acetic acid (0·583 M), spiked with 20 µl rhamnetin (internal standard 50 µg/ml in methanol) and subsequently treated with a mixture of 10^4 units β-glucuronidase/$2·5 \times 10^2$ units sulphatase (crude extract from *Helix pomatia*). After an incubation of 1 h at 37°C, the flavonol aglyca quercetin, kaempferol, isorhamnetin and tamarixetin were simultaneously extracted with 5·5 ml acetone. The mixture was centrifuged at 3700 g for 45 min and the supernatant evaporated under vacuum at 45°C to dryness. The residue was resolved in 200 µl methanol, 77·5 µl nanopure H_2O and 22·5 µL HCl (10 mol/l).

For HPLC analysis, 30 µl were injected onto a C-18 Kromasil 100 column (dimensions 250×4 mm, particle size 5 µm) guarded by a C-18 Inertsil ODS-2 precolumn (Jasco, Groß-Umstadt, Germany). The eluent (1 ml/min) was composed of 0·025 mol/l NaH_2PO_4, pH 2·4, acetonitrile and methanol (68:27:5 v/v/v). The column effluent was mixed with 0·4 ml/min 1·0 mol/l $Al(NO_3)_3$ in methanol containing 7·5 % (v/v) acetic acid in a post-column reactor (Jasco). The column and the reactor were placed in a column oven at 30°C. The fluorescence of the ensuing flavonol-metal complex was measured at 485 nm using a fluorescence detector with an excitation wavelength of 422 nm. The limit of detection was 3 ng/ml.

Statistics

All statistical analyses were performed using SPSS for windows version 10.0 (SPSS, Chicago, IL, USA). Data were checked for normality and log (base 10) transformed if necessary. Univariate ANOVA, two-factor within-subjects ANOVA with Tukey as a *post hoc* test, Pearson's correlations and paired-sample Student's t tests were used; P values $\leq 0·05$ were taken as significant. In all graphs, data are presented as mean values (six subjects) with their standard errors of the mean unless stated otherwise.

Results

Flavonoid composition of the high- and low-quercetin soups

The flavonoid concentrations of the soups derived from high- and low-quercetin onions were determined by HPLC analysis (Table 1). From the non-hydrolysed extracts, it appeared that, after cooking, 99 % of the total quercetin concentration was still present as glycosides, in a ratio of quercetin 4'-glucoside to quercetin 3,4'-glucoside of 0·88:1, for both the low- and high-quercetin soups. This ratio is in agreement with the previous findings of de Pascual-Teresa *et al.* (2004). Thus, in the present study, each subject consumed 600 ml soup containing a total amount of 68·8 or 4·9 mg quercetin, for the high- and low-quercetin soups, respectively.

Plasma flavonol concentrations following ingestion of onion soup

Following ingestion of the high-quercetin and low-quercetin soups, the concentrations of quercetin and two of its metabolites with an intact flavonol structure, isorhamnetin and tamarixetin, were quantified in blood plasma. At 90 min after ingesting the high-quercetin soup, mean plasma quercetin concentrations peaked at 2·59 (SEM 0·42) µmol/l

($P=0.0001$; two-factor within-subjects ANOVA; Fig. 1A), and then decreased steadily, returning to basal levels after 32 h. The mean area under the curve for the high-quercetin soup plasma profile was 911·61 (SEM 85·17) μmol/l per min, significantly higher than the mean area under the curve for the low-quercetin soup plasma profile ($P=0.001$; paired Student's t test). Plasma quercetin levels after low-quercetin soup ingestion were negligible.

Peak plasma isorhamnetin concentrations occurred 2 h after ingestion of the quercetin soups at 0·119 (SEM 0·02) μmol/l and 0·0133 (SEM 0·04) μmol/l ($P=0.0001$; two-factor within-subjects ANOVA) for the high- and low-quercetin soups respectively (Fig. 1B). Plasma isorhamnetin levels thereafter declined slowly, returning to basal levels by 32 h. Peak plasma concentrations of tamarixetin occurred 2·5 h after ingestion of the soups at 0·172 (SEM 0·035) μmol/l and 0·0049 (SEM 0·001) μmol/l ($P=0.0001$; two-factor within subjects ANOVA) for the high- and low-quercetin soups respectively (Fig. 1C). Plasma levels declined thereafter with very similar kinetics to those of isorhamnetin.

The sum of the plasma concentrations of the three flavonoids is shown in Fig. 1D. Total flavonoid concentrations peaked at 90 min and were 2·88 (SEM 0·47) μmol/l and 0·122 (SEM 0·122) μmol/l ($P=0.0001$; two-factor within-subjects ANOVA) for the high- and low-quercetin soups, respectively. At all time points from 30 to 420 min after soup ingestion, significantly higher concentrations of total flavonols, quercetin and metabolites (tamarixetin, isorhamnetin) were observed after the high-quercetin soup than after the low-quercetin soup and when compared with baseline concentrations ($P=0.0001$; two-factor within subjects ANOVA with Tukey as a *post hoc* test).

High-quercetin soup ingestion inhibits collagen-stimulated platelet aggregation

Ingestion of the high-quercetin soup inhibited platelet aggregation (*ex vivo*) at 1 and 3 h after ingestion (Fig. 2) compared with baseline and when corrected for the effect of low-quercetin soup ingestion on platelet aggregation. The inhibitory effect was greater 3 h after ingestion of the high-quercetin soup, when platelets were stimulated with 0·5, 1 and 2 μg/ml collagen. Indeed, the inhibition of platelet aggregation was dependent on the concentration of collagen used to stimulate aggregation, with higher concentrations of collagen overcoming the inhibitory effect of the treatment. Platelet aggregation was adjusted for the level of response measured following ingestion of the low-quercetin soup. Although the trends observed were quite dramatic, they did not achieve statistical significance in this assay, possibly owing to the natural variability of platelet responses to collagen in different individuals and the small subject number possible in this pilot study. There was little effect on platelet aggregation following ingestion of the low-quercetin soup with a mean increase of 9·6 (SEM 5·4) %.

High-quercetin soup ingestion inhibits collagen-stimulated signalling in platelets

Owing to the potent inhibitory activity of quercetin on platelet aggregation and signalling *in vitro* (Hubbard *et al.* 2003) and the inhibitory effect of high-quercetin soup ingestion on platelet aggregation *ex vivo* (Fig. 2), the effect of high-quercetin soup ingestion on collagen-stimulated signalling was investigated.

In our previous study (Hubbard *et al.* 2004), inhibition of the tyrosine phosphorylation of critical proteins in the GPVI signalling pathway, namely Syk and PLCγ2, was observed
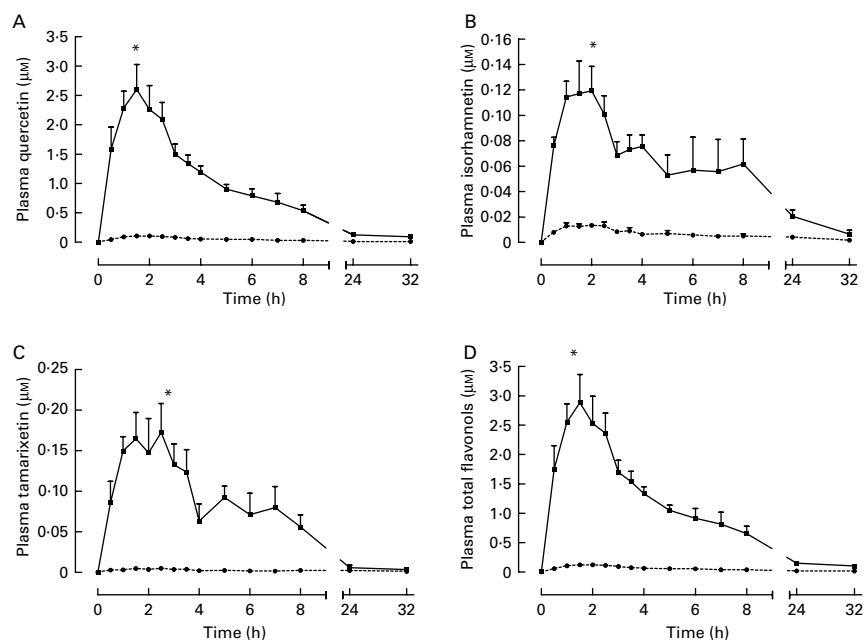

Fig. 1. The effect of ingestion of high-quercetin (■) or low-quercetin (●) soup on plasma concentrations of (A) quercetin, (B) isorhamnetin, (C) tamarixetin and (D) total of the three flavonols. Subjects ingested 600 ml high- or low-quercetin soup. Blood samples were taken into K3 EDTA tubes and centrifuged, and the plasma was frozen at −80°C. Plasma flavonol concentrations were derived by HPLC analysis with fluorescence detection. The concentration of flavonols in plasma is shown for a 32 h period (*n* 6; mean values with their standard errors *$P=0.0001$). At all time points from 30 to 420 min, significantly higher concentrations of total flavonols, quercetin and metabolites (tamarixetin and isorhamnetin) were observed after ingestion of the high-quercetin soup than after ingestion of the low-quercetin soup and when compared with baseline concentrations ($P=0.0001$; two-factor within-subjects ANOVA with Tukey as a *post hoc* test).

upon ingestion of a purified quercetin-containing drink. In the present study, differential effects of the quercetin soup ingestion were observed. When the low-quercetin soup was ingested, collagen-stimulated Syk tyrosine phosphorylation increased compared with baseline at 1 and 3 h, whereas upon ingestion of the high-quercetin soup, collagen-stimulated Syk tyrosine phosphorylation was inhibited at 1 and 3 h compared with baseline and reached statistical significance at 1 h ($P=0.003$; ANOVA with Tukey as a *post hoc* test) (Fig. 3). The inhibition of Syk tyrosine phosphorylation upon high-quercetin soup ingestion was time dependent, a greater effect being observed 3 h after soup ingestion. Syk tyrosine phosphorylation was negatively correlated with the area under the curve for the high-quercetin plasma profile at both 1 and 3 h ($P=0.022$, $r-0.650$, and $P=0.007$, $r-0.734$ for the 1 h and 3 h time points respectively, and $P=0.0001$, $r-0.699$ for the 1 and 3 h values together (total; Pearson's correlation).

Similarly collagen-stimulated tyrosine phosphorylation of PLCγ2 also increased upon low-quercetin soup ingestion, compared with baseline at 1 and 3 h, whereas upon ingestion of the high-quercetin soup, collagen-stimulated PLCγ2 tyrosine phosphorylation was inhibited at 1 and 3 h compared with baseline. Both these effects were less potent with time (Fig. 4).

Discussion

The inhibitory effect of dietary flavonoids on platelet function has been recognised for some time, but controversy surrounds the proposition that this may underlie some of the protective effects of a diet rich in flavonoids. Indeed, it has been suggested that quercetin is poorly absorbed through the gut and therefore does not reach the plasma at levels that affect platelet function. We have, however, recently reported the identity of specific targets of collagen-mediated signalling pathways that lead to platelet activation and are inhibited by quercetin *in vitro*; these include src-family kinases, the tyrosine kinase Syk and phosphoinositide 3-kinase (Hubbard *et al.* 2003). These inhibitory effects were mirrored *ex vivo* upon ingestion of quercetin 4′-glucoside in the form of a supplement drink (Hubbard *et al.* 2004).
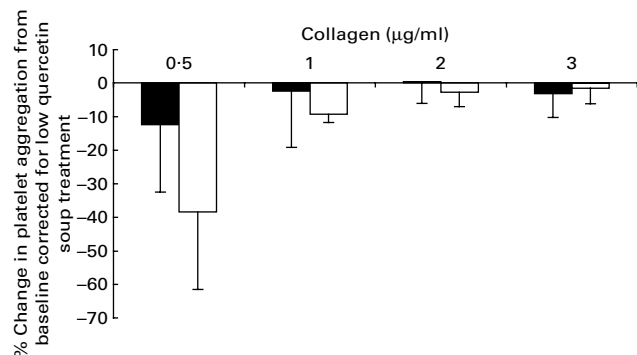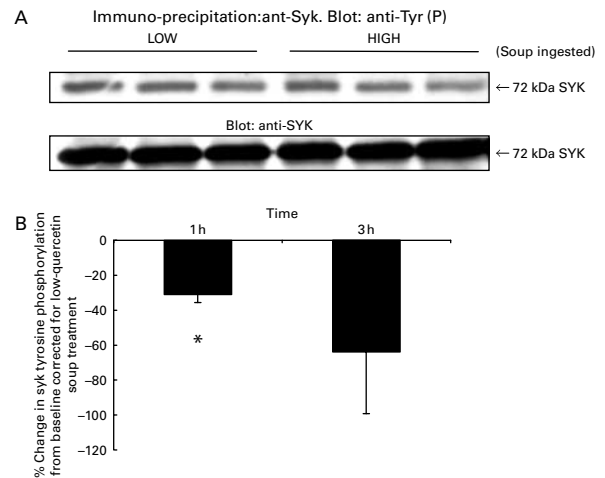

Fig. 3. The effect of onion soup ingestion on tyrosine phosphorylation of Syk in platelets. Subjects ingested 600 ml high- or low-quercetin soup. Washed platelets were prepared 0, 1 and 3 h after ingestion of the soup. Platelets were then stimulated under non-aggregating conditions with collagen (25 μg/ml) for 90 s. Platelets were then lysed, and Syk was immunoprecipitated and separated by SDS-PAGE, followed by immunoblotting for phosphotyrosine residues, and then reprobed for equal loading of Syk. (A) Representative phosphotyrosine blot. (B) Densitometry analysis of Syk tyrosine phosphorylation of all subjects compared with baseline (0 h) values (*n* 6; mean values with their standard errors; **$P=0.003$ high-quercetin compared with low-quercetin soup).

Recent reports demonstrate that quercetin bioavailability is highly dependent on the form of quercetin that is ingested. Quercetin 4′-glucoside has been reported to be preferentially absorbed into the body from the gut in comparison to other forms of quercetin, such as quercetin 3-O-β-rutinoside
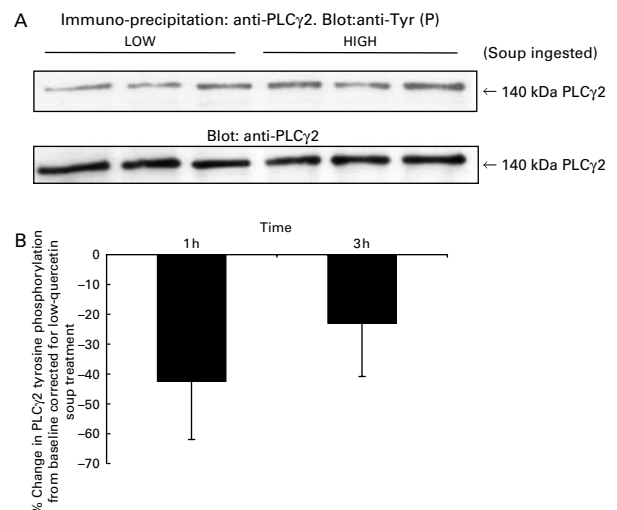

Fig. 4. The effect of onion soup ingestion on tyrosine phosphorylation of phospholipase Cγ2 (PLCγ2) in platelets. Subjects ingested 600 ml high- or low-quercetin soup. Washed platelets were prepared from whole blood 0 (before soup ingestion), 1 and 3 h after ingestion of the soup. Platelets were then stimulated under non-aggregating conditions with collagen (25 μg/ml) for 90 s. Platelets were then lysed, and PLCγ2 was immunoprecipitated and separated by SDS-PAGE, followed by immunoblotting for phosphotyrosine residues, and then reprobed for equal loading of PLCγ2. (A) Representative phosphotyrosine blot. (B) Densitometry analysis of PLCγ2 tyrosine phosphorylation of all subjects compared with baseline (0 h) values (*n* 6; standard error of the mean).

Fig. 2. High-quercetin soup ingestion inhibits collagen-induced platelet aggregation 1 and 3 h after consumption compared with baseline. Washed platelets were prepared at 0 (before soup ingestion), 1 (■) and 3 (□) h after ingestion of the soup. The platelets were stimulated with concentrations of collagen, and the aggregation response was monitored for 90 s. Percentage change in platelet aggregation compared with baseline and corrected for low-quercetin soup is shown for four concentrations of collagen (*n* 6; standard error of the mean).

(Hollman *et al.* 1999; Hubbard *et al.* 2004), with peak plasma quercetin concentrations reported in the range $5-10\,\mu$M. In experiments previously described (Hubbard *et al.* 2003, 2004), quercetin inhibited collagen-stimulated cell signalling and platelet aggregation *in vitro* and *ex vivo* at similar concentrations.

The aims of the present study were to explore the bioavailability of quercetin from a dietary source such as onion soup, and to determine whether the levels adsorbed into blood plasma reached levels that were able to inhibit collagen-mediated signalling in platelets and thereby reduce platelet aggregation responses. Published estimates of the daily dietary intake of flavonoids vary considerably, with reports of 26 mg/d for flavonols (a subgroup of flavonoids; Hertog *et al.* 1995) to 1·26 g/d total phenolics in a group of vegetarian women (Gosnay *et al.* 2002). The dose of quercetin supplied by the high-quercetin soup (69 mg total quercetin) was chosen as a high dose of dietary quercetin compared with population estimates (Hertog *et al.* 1995), but one that could be achieved by dietary means. The dose of quercetin supplied by the low-quercetin soup (4 mg total quercetin) was 17-fold lower and represented a low dose compared with population estimates (Hertog *et al.* 1995; Gosnay *et al.* 2002).

Following ingestion of the high- and low-quercetin soups, analysis of the quercetin concentration in plasma revealed that quercetin was absorbed from the gut into the bloodstream after ingestion of both soups, but peak quercetin levels were over 150-fold higher after ingestion of the high-quercetin soup. The concentration of quercetin measured in plasma samples encompassed all forms of quercetin (total quercetin). The plasma samples were enzymically treated to yield free quercetin, allowing no differentiation between conjugates such as glucuronides and sulphates, which are difficult to identify in plasma, owing to the lack of standards. The peak plasma concentrations of the methylated metabolites of quercetin, namely isorhamnetin and tamarixetin, were approximately 20- and 15-fold lower, respectively, than those of quercetin after high-quercetin soup ingestion. Isorhamnetin and tamarixetin were also measured in plasma following enzymic treatment; thus, the aglycone form of these compounds was not detected in plasma. These compounds are therefore thought to be present in blood plasma in conjugate forms such as glucuronides or sulphates. The parallel absorption and elimination profiles of all three compounds detected suggests that the methylated metabolites isorhamnetin and tamarixetin were derived from the intestinal and/or hepatic metabolism of quercetin, as suggested by Manach *et al.* (1996) and Ader *et al.* (2000).

The conjugates of quercetin could not be detected in plasma, so the identity of the dominant or bioactive conjugate form of quercetin was not addressed in this study. The onion soups have been shown to contain the quercetin aglycone, quercetin 4′-O-β-D-glucoside and quercetin 3, 4′-glucoside (de Pascual-Teresa *et al.* 2004), but which of these quercetin derivatives or of their metabolites is the active form is an important issue to resolve.

If the peak plasma quercetin concentrations from the quercetin-supplementation study (Hubbard *et al.* 2004) are compared with the results observed in the present study, a dose-dependent effect can be seen. In the present study, 69 mg total quercetin (of which 32 mg was quercetin 4′-glucoside) in the form of onion soup was fed to subjects, producing a plasma peak of $2·59\,\mu$M. In the previous supplementation study, 150 and 300 mg purified quercetin 4′-glucoside were ingested by subjects, producing plasma peaks of 4·66 and 9·72 μmol/l, respectively.

Collagen-stimulated platelet aggregation was inhibited by ingestion of the high-quercetin soup. The inhibitory effect was dependent on the concentration of collagen used to stimulate platelet aggregation, consistent with previous *in vitro* and *ex vivo* data (Hubbard *et al.* 2003, 2004). The inhibition of platelet aggregation by collagen after ingestion of the high-quercetin soup was also time-dependent, more potent effects being seen 3 h after ingestion of the soup compared with 1 h.

Very few studies have investigated the acute effects of dietary supplementation of flavonoids on platelet function. Studies investigating the effects of black tea ingestion on ADP and collagen-induced platelet aggregation have reported little effect (Duffy *et al.* 2001; Hodgson *et al.* 2002). The chronic ingestion of polyphenol-rich foods has been observed to inhibit platelet aggregation by Keevil *et al.* (2000), who supplemented ten subjects with purple grape juice for 7 d; this inhibited whole-blood platelet aggregation induced with collagen (1 μg/ml) by 77 %. Other similar studies have, however, reported no significant inhibitory effect of the ingestion of polyphenol-rich foods on platelet aggregation (Conquer *et al.* 1998; Janssen *et al.* 1998). A number of these studies used high concentrations of agonist to stimulate platelet aggregation. Janssen *et al.* (1998) used collagen as an agonist at 2·0 μg/ml, and Conquer *et al.* (1998) used collagen at 10 μg/ml. In the present study, the inhibition of platelet aggregation by ingestion of the high-quercetin soup was more potent with the lower concentrations of collagen (0·5 and 1·0 μg/ml). It is likely that discrepancies between these studies are attributable to a combination of factors including potencies of agonists, sources of polyphenols, other dietary factors and donor variability in platelet responses.

In the present study, tyrosine phosphorylation of the tyrosine kinase Syk and phospholipase Cγ2 on stimulation with collagen were inhibited by ingestion of the high-quercetin soup. This evidence supports previous *in vitro* and *ex vivo* evidence presented by the authors (Hubbard *et al.* 2003, 2004) and indicates that quercetin obtained in the diet is capable of reducing GPVI signalling in platelets and therefore suppressing platelet reactivity. The inhibitory effect of quercetin on collagen-stimulated platelet activation via GPVI observed *ex vivo* occurs therefore, at least in part, by the same mechanisms as the *in vitro* addition of quercetin to platelets (Hubbard *et al.* 2003). The inhibition of PLCγ2 tyrosine phosphorylation by quercetin ingestion was less than that of Syk and was not found to be statistically significant. PLCγ2 tyrosine phosphorylation can occur via signalling pathways in addition to that of GPVI, so interference from other pathways may have masked detection of the inhibition of PLCγ2 tyrosine phosphorylation. Collagen-stimulated Syk tyrosine phosphorylation was negatively correlated with the area under the curve for the high-quercetin soup plasma profile. This correlation suggests a major role for Syk tyrosine phosphorylation in the inhibition of platelet function by quercetin ingestion.

In recent years, a number of dietary sources of inhibitors of platelet function have been reported, although the relationship between diet, platelet function and pathology remains unresolved. This area of research has been largely prompted by epidemiological data, suggesting that diet and CVD may be linked. The evidence presented here not only suggests that quercetin may play an important role in health and disease regarding the inhibition of platelet function and hence a possible decreased risk of thrombosis, but also highlights the potential role of

quercetin as an inhibitor of a number of cell-signalling mechanisms *in vivo*. The discovery that the effects of quercetin on cell-signalling mechanisms *ex vitro* can be reproduced after ingestion of quercetin as a human dietary supplement (Hubbard *et al.* 2004) and from a dietary source such as onion soup may have repercussions for the role of quercetin in a number of disease states, including heart disease and cancer. The evidence presented here also further substantiates the epidemiological data suggesting that those who preferentially consume high amounts of apples, onions, red wine and tea containing high concentrations of quercetin have a reduced risk of CVD (Hertog *et al.* 1993, 1995; Hollman *et al.* 1996).

Acknowledgements

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