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ORIGINAL ARTICLE

Ingestion of quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in humans

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Summary. *Background*: Quercetin, a flavonoid present in the human diet, which is found in high levels in onions, apples, tea and wine, has been shown previously to inhibit platelet aggregation and signaling *in vitro*. Consequently, it has been proposed that quercetin may contribute to the protective effects against cardiovascular disease of a diet rich in fruit and vegetables. *Objectives*: A pilot human dietary intervention study was designed to investigate the relationship between the ingestion of dietary quercetin and platelet function. *Methods*: Human subjects ingested either 150 mg or 300 mg quercetin-4′-O-β-D-glucoside supplement to determine the systemic availability of quercetin. Platelets were isolated from subjects to analyse collagen-stimulated cell signaling and aggregation. *Results*: Plasma quercetin concentrations peaked at 4.66 μM (± 0.77) and 9.72 μM (± 1.38) 30 min after ingestion of 150-mg and 300-mg doses of quercetin-4′-O-β-D-glucoside, respectively, demonstrating that quercetin was bioavailable, with plasma concentrations attained in the range known to affect platelet function *in vitro*. Platelet aggregation was inhibited 30 and 120 min after ingestion of both doses of quercetin-4′-O-β-D-glucoside. Correspondingly, collagen-stimulated tyrosine phosphorylation of total platelet proteins was inhibited. This was accompanied by reduced tyrosine phosphorylation of the tyrosine kinase Syk and phospholipase Cγ2, components of the platelet glycoprotein VI collagen receptor signaling pathway. *Conclusions*: This study provides new evidence of the relatively high systemic availability of quercetin in the form of quercetin-4′-O-β-D-glucoside by supplementation, and implicates quercetin as a dietary inhibitor of platelet cell signaling and thrombus formation.

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Introduction

Platelets play a pivotal role in both health and disease, through their central involvement in hemostasis and thrombosis. Platelet activation in response to collagen is mediated principally through collagen binding to the platelet receptor glycoprotein VI (GPVI) [1]. Ligation and clustering of GPVI results in the tyrosine phosphorylation of the associated transmembrane protein the Fc receptor γ-chain within its immunoreceptor tyrosine-based activation motif (ITAM) [2,3]. This leads to the assembly of a complex of signaling proteins at the plasma membrane and the activation of a number of cell-signaling pathways [4,5]. These include activation of Syk, phospholipase Cγ2 (PLCγ2) and phosphoinositide 3-kinase (PI3-K)-dependent signaling mechanisms, the actions of which are essential for platelet aggregation [5,6].

Platelet function is heavily implicated in the development of thrombosis, and indeed the targeting of platelets with drugs that specifically inhibit platelet function has proven to be beneficial in the prevention of thrombosis in some groups of patients [7,8].

The relationship between a diet high in fruit and vegetables and a decreased risk of thrombosis is well established, although an explanation for this is far from clear [9,10]. A number of studies have focused on the effects of dietary components on platelet function, and among these are the flavonoids. Quercetin (and its conjugate forms) is a major flavonoid in the human diet, and is found in many fruits and vegetables, with high levels found in onions, apples, tea and wine [11]. One of the major conjugates, quercetin-4′-O-β-D-glucoside (Q-4-G) is present in a number of fruits and vegetables and can be found in onions at particularly high levels. This form has been found to be

preferentially absorbed in the gut in human studies involving single meals [12] or supplementation with purified compound [13,14], where plasma quercetin levels up to 3.5 μM have been detected.

We have reported recently that quercetin inhibits potently collagen-stimulated platelet aggregation through the inhibition of GPVI-mediated signaling *in vitro*, whereas more modest inhibition of thrombin and ADP-induced aggregation was observed [15]. Quercetin was shown to inhibit platelet aggregation and the tyrosine phosphorylation and/or kinase activity of a number of critical components of the GPVI signaling pathway. These include the non-receptor tyrosine kinase Syk, PLCγ2 and PI3-K. The IC_{50} values for the inhibition of platelet aggregation by quercetin were below 3 μM when platelets were stimulated with concentrations of collagen between 0.5 and 1.0 μg mL^{-1} [15].

In the current study we investigate the potential inhibitory effects of quercetin ingestion on collagen-stimulated platelet aggregation and signaling, by supplementing subjects with a single dose of Q-4-G.

Methods

Materials

Beta-glucuronidase/sulphatase (crude extract from *Helix pomatia*) and protein A-sepharose 4BL (PAS) were purchased from Sigma (Poole, UK). Horm-Chemie collagen (collagen-fibres from equine tendons) was purchased from Nycomed (Munich, Germany). Antiphosphotyrosine monoclonal antibody (4G10) (1 μg mL^{-1}) was from Upstate Biotechnology (TCS Biologicals, Botolph Claydon, UK). Anti-Syk (N-19), anti-Syk (LR) and anti-PLCγ2 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Autogen Bioclear UK Ltd, Calne, UK). Horseradish peroxidase (HRP)-conjugated secondary antibodies (antirabbit IgG, antimouse IgG at 1 : 10 000), the enhanced chemiluminescence immunodetection system and Hyperfilm were purchased from Amersham Biosciences (Little Chalfont, UK). All chemicals used for preparing the high-performance liquid chromatography (HPLC) eluent were purchased from Roth (Karlsruhe, Germany). Quercetin-4′-O-β-D-glucoside (Q-4-G) (97% by HPLC) was from Polyphenols AS (Sandnes, Norway). Ethanol (99.7%) was from Rathburn Chemicals Ltd (Walkerburn, UK).

Human supplementation study design

Approval from the University of Reading Ethics Committee was obtained with all subjects giving informed consent before commencement of the study. In a three-treatment cross-over study, six healthy subjects (three male and three female) who had abstained from ingesting aspirin for at least 2 weeks prior to the study complied with a quercetin-low diet for 14 days (foods low in quercetin were defined according to Hertog *et al.* [11]), to prevent the influence of quercetin from normal dietary sources. The current study was designed on the basis of an investigation by Hollman *et al.* [13], who fed subjects 160 mg Q-4-G in the form of a drink. By comparison, the subjects in the current study ingested either 150 mg (296 μmol) or 300 mg (592 μmol) Q-4-G solubilized in 5% (v/v) ethanol, or a 5% (v/v) ethanol control drink, the order of which was randomized. Blood samples were taken through the course of the study day and at 24 and 32 h. Blood samples taken at 0, 30 and 120 min were also used to examine the effect of supplementation on platelet function and signaling. The study day was followed by a 1-month washout period, after which the protocol was repeated for remaining treatments.

In a study by Ferry *et al.* [16], subjects were injected with quercetin intravenously. Approximately 3000 mg quercetin was required to produce a toxic response via this route of administration. No toxic effects of quercetin ingestion were observed in the current study.

Preparation and stimulation of platelets

Platelets were prepared at 0, 30 and 120-min time points on the day of the experiment by differential centrifugation as described previously [5]. Stimulation of platelets (450 μL) with collagen (50 μL) (0.5, 1, 2, 3, 4 and 5 μg mL^{-1} final concentration) was performed at 37 °C in an optical platelet aggregometer (Chrono-log Corp., Havertown, PA, USA). For immunoblotting experiments, platelets were suspended to 8×10^8 cells mL^{-1} with 1 mM EGTA to prevent aggregation. The concentration of collagen was increased to 25 μg mL^{-1} to enable the detection of tyrosine phosphorylation events. Tyrosine phosphorylation was undetectable at lower concentrations of collagen, consistent with previous reports [3,15]. Platelet activation and immunoprecipitation were performed as described previously [15]. Antibodies to Syk and PLCγ2 were used at a concentration of 1 μg mL^{-1}.

Immunoblotting

Immunoblotting was carried out as previously described [5]. Densitometry analysis was carried out using a BioRad GS710 densitometer with Quantity One analysis software (BioRad, Hemel Hempstead, UK).

Plasma concentrations of quercetin, isorhamnetin and tamarixetin

Plasma concentrations of flavonoids were measured by HPLC analysis with fluorescence detection as described previously [17,18].

Statistics

All statistical analyses were performed using SPSS for Windows version 10.0 (SPSS, Chicago, IL, USA). Univariate analysis of variation (ANOVA), two factor within subjects ANOVA and paired sample *t*-tests (Student) were used and *P*-values ≤ 0.05 were taken as significant. In all graphs data are

presented as mean values (six subjects) with ± standard error of mean (SEM) unless stated otherwise.

Results

The systemic availability of quercetin following supplementation with quercetin 4′-O-β-D-glucoside

Following ingestion of Q-4-G (150-mg and 300-mg doses), the concentrations of quercetin and two of its metabolites with an intact flavonol structure, isorhamnetin and tamarixetin, were quantified in blood plasma. Thirty minutes after ingestion of the 150-mg and the 300-mg doses of Q-4-G, plasma quercetin concentrations peaked at 4.66 μM (± 0.77) and 9.72 μM (± 1.38), respectively (Fig. 1A). Plasma quercetin concentrations then decreased dramatically, plateaued after 2 h and returned to basal levels after 32 h.

Peak plasma isorhamnetin concentrations also occurred 30 min after ingestion of Q-4-G at 0.16 μM (± 0.05) and 0.44 μM (± 0.07) for the 150-mg and 300-mg doses, respectively (Fig. 1B). The plasma concentrations of tamarixetin peaked 30 min (0.24 ± 0.07 μM) and 45 min (0.54 ± 0.09 μM) after ingestion of the 150-mg and 300-mg doses of Q-4-G, respectively (Fig. 1C). The plasma profiles of isorhamnetin and tamarixetin thereafter declined with very similar kinetics to quercetin.

The sum of the plasma concentrations of the three flavonoids is shown in Fig. 1D. Total flavonoid concentrations peaked at 30 min and were 5.07 μM (± 0.90) and 10.66 μM (± 1.55) for the 150-mg and 300-mg doses of Q-4-G, respectively, indicating dose-dependent systemic availability of these flavonoids by supplementation.

Quercetin 4′-O-β-D-glucoside supplementation inhibits collagen-induced platelet aggregation

Ingestion of 150 mg Q-4-G inhibited platelet aggregation (*ex vivo*) 30 min after ingestion at all concentrations of collagen tested (Fig. 2A) compared with baseline, reaching statistical significance with 0.5 μg mL^{-1} collagen ($P = 0.001$). This inhibitory effect was greater 120 min after ingestion of the drink, where it reached statistical significance with 0.5, 1 and 2 μg mL^{-1} collagen ($P = 0.001$). Indeed, the inhibition of platelet aggregation by 150 mg Q-4-G was dependent on the concentration of collagen used to stimulate aggregation, with higher concentrations of collagen overcoming the inhibitory effect of the treatment. Ingestion of 300 mg Q-4-G also caused inhibition of platelet aggregation 30 min after ingestion with all concentrations of collagen compared with baseline (Fig. 2B), reaching statistical significance with 0.5 μg mL^{-1} collagen ($P = 0.001$). Thirty minutes after

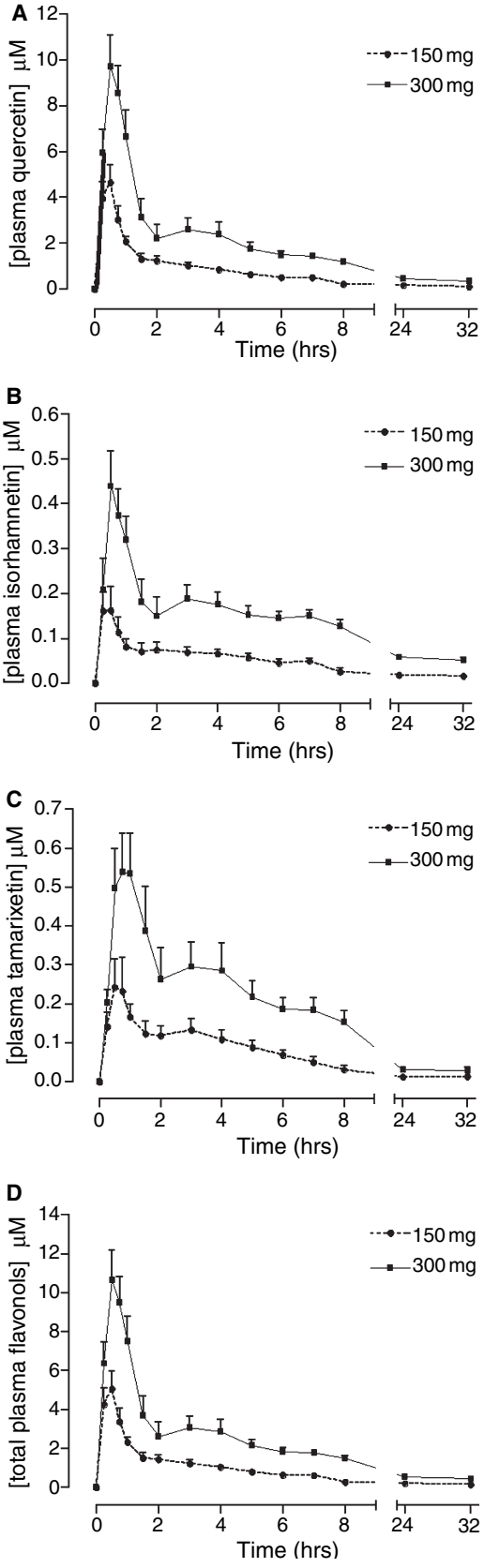

Fig. 1. Plasma concentration–time curve of (A) quercetin, (B) isorhamnetin, (C) tamarixetin and (D) total of the three flavonols following supplementation with quercetin-4′-O-β-D-glucoside (Q-4-G). Subjects ingested 150 mg or 300 mg of Q-4-G. Blood samples were taken, centrifuged and the plasma frozen at −80 °C. Plasma quercetin concentrations were derived by high-performance liquid chromatography analysis with fluorescence detection ($n = 6$ ± SEM).

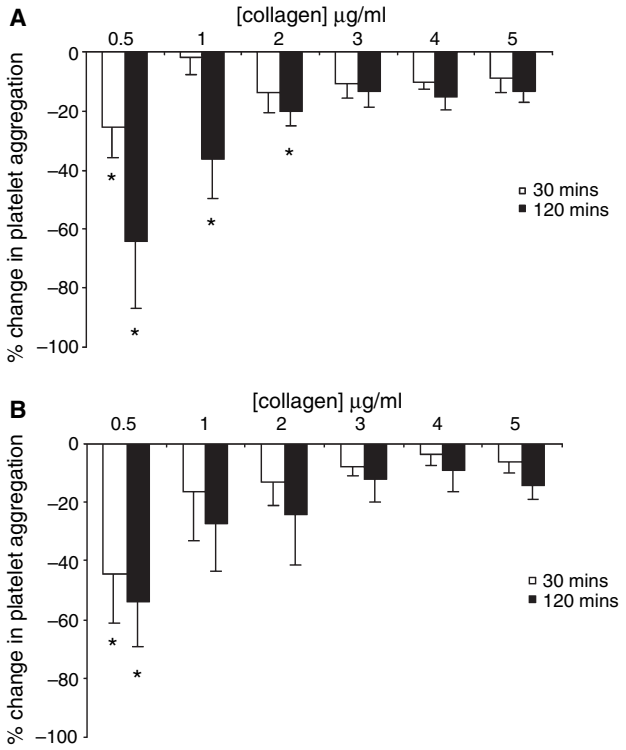

Fig. 2. Quercetin 4′-O-β-D-glucoside (Q-4-G) supplementation inhibits collagen-induced platelet aggregation, 30 and 120 min after consumption compared with baseline. Washed platelets were prepared at 0 (before drink ingestion), 30 and 120 min after ingestion of (A) 150 mg and (B) 300 mg Q-4-G, or control drink. The platelets were stimulated with collagen and aggregation responses monitored. Percentage change in platelet aggregation compared with baseline and corrected for control is shown for six concentrations of collagen ($n = 6 \pm$ SEM, $^*P = 0.001$ compared with baseline).

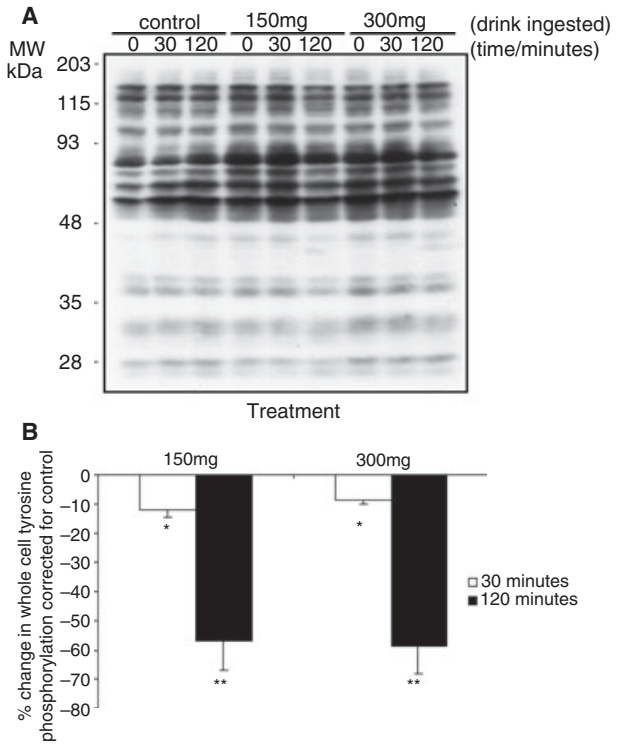

Fig. 3. Quercetin 4′-O-β-D-glucoside (Q-4-G) supplementation inhibits whole platelet protein tyrosine phosphorylation. Subjects ingested 150 mg or 300 mg Q-4-G, or control drink. Platelets were prepared, 0 (before ingestion), 30 and 120 min after ingestion of the drink and stimulated under non-aggregating conditions with collagen (25 μg mL^{-1}) for 90 s. Platelets were lysed and proteins separated by SDS–PAGE, followed by immunoblotting for protein tyrosine phosphorylation. (A) A representative blot for the three treatment groups and 0, 30, and 120-min time points for each group. (B) Percentage change in total whole platelet protein tyrosine phosphorylation measured by densitometry compared with baseline (0 time point) values and corrected for control ($n = 6 \pm$ SEM; $^*P = 0.001$ compared with baseline; $^{**}P = 0.001$ compared with baseline and 30 min).

ingestion, the inhibition of platelet aggregation with 0.5 μg mL^{-1} collagen was 2-fold greater than for 150 mg Q-4-G. The inhibition of platelet aggregation was maintained after 120 min with 0.5 μg mL^{-1} ($P = 0.001$). Platelet aggregation was adjusted for the level of response measured following ingestion of the control drink. In most individuals the control drink had no effect on aggregation responses, although an occasional modest increase in responsiveness was observed in some subjects (data not shown).

Quercetin 4′-O-β-D-glucoside supplementation inhibits collagen-stimulated signaling in platelets

Due to the potent inhibitory activity of quercetin on platelet aggregation and signalling *in vitro* [15] and the inhibitory effect of the ingestion of Q-4-G on platelet aggregation *ex vivo* (Fig. 2), the effect of the ingestion of Q-4-G on collagen-stimulated platelet signaling was investigated. Platelet activation is associated with rapid phosphorylation of tyrosine residues present in a number of intracellular molecules [4]. Figure 3A shows a representative phosphotyrosine immunoblot of whole-cell lysates from platelets following stimulation with collagen for each treatment for one subject. In both the 150-mg and 300-mg Q-4-G treatment groups, the tyrosine phosphorylation of a number of proteins in the whole-cell lysates decreased with time, in comparison with levels before supplementation (0 time point). The tyrosine phosphorylation of a number of proteins in the control samples increased with time following ingestion of the control drink. Densitometry analysis of the corresponding immunoblots from all six subjects is shown in Fig. 3B. After ingestion of 150 mg and 300 mg Q-4-G drink, whole-cell tyrosine phosphorylation decreased significantly ($P = 0.001$) in a time-dependent manner to similar degrees, 30 and 120 min after ingestion when compared with baseline values (Fig. 3B).

The potent inhibitory effect of Q-4-G ingestion on whole-cell tyrosine phosphorylation led to further investigations of critical proteins in the GPVI signaling pathway. The effect of Q-4-G drink ingestion on the collagen-stimulated tyrosine phosphorylation of Syk and PLCγ2 was investigated. A representative Syk phosphotyrosine immunoblot is shown in Fig. 4A. Tyrosine phosphorylation of Syk was inhibited 30 and 120 min after ingestion of 150 mg and 300 mg of Q-4-G. This

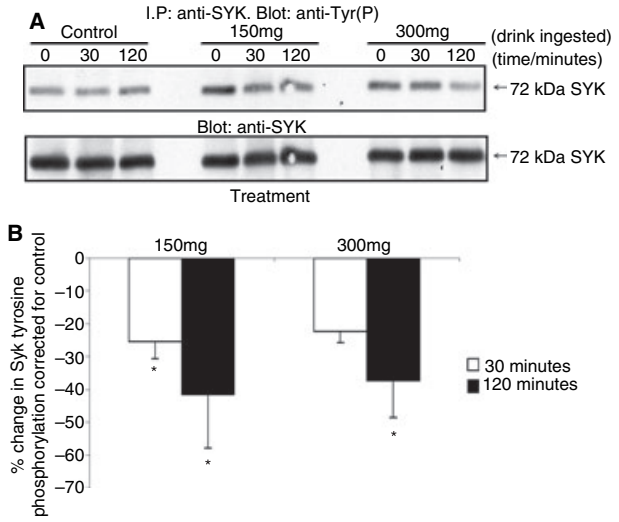

Fig. 4. Quercetin 4′-O-β-D-glucoside (Q-4-G) supplementation inhibits tyrosine phosphorylation of Syk in platelets. Subjects ingested 150 mg or 300 mg Q-4-G, or control drink. Washed platelets were prepared, 0 (before drink ingestion), 30 and 120 min after ingestion of the drink. Platelets were then stimulated under non-aggregating conditions with collagen (25 μg mL^{-1}) 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) A representative phosphotyrosine blot. (B) Densitometry analysis of Syk tyrosine phosphorylation of all subjects compared with baseline (0 time point) values and corrected for control ($n = 6 \pm$ SEM, $^*P = 0.05$ compared with baseline).

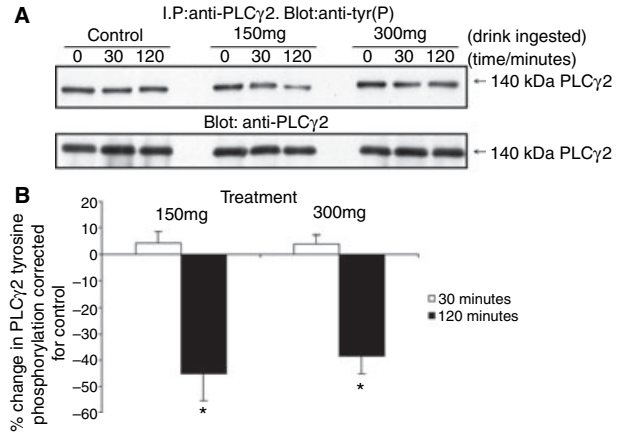

Fig. 5. Quercetin 4′-O-β-D-glucoside (Q-4-G) supplementation inhibits tyrosine phosphorylation of phospholipase Cγ2 (PLCγ2) in platelets. Subjects ingested 150 mg or 300 mg Q-4-G, or control drink. Washed platelets were prepared, 0 (before drink ingestion), 30 and 120 min after ingestion of the drink. Platelets were then stimulated under non-aggregating conditions with collagen (25 μg mL^{-1}) 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) A representative phosphotyrosine blot. (B) Densitometry analysis of PLCγ2 tyrosine phosphorylation of all subjects compared with baseline (0 time point) values and corrected for control ($n = 6 \pm$ SEM, $^*P = 0.05$ compared with baseline).

is further illustrated by densitometry analysis of Syk tyrosine phosphorylation for all six subjects, compared with baseline (0 time point) and corrected for control (Fig. 4B). Collagen-stimulated tyrosine phosphorylation of Syk was inhibited significantly ($P = 0.05$) in a time-dependent manner by ingestion of both 150 mg and 300 mg of Q-4-G drink. Furthermore, 120 min after ingestion of both the 150-mg and 300-mg Q-4-G drinks, Syk tyrosine phosphorylation was inhibited by 41.5% (SEM ± 16.25) and 37.2% (SEM ± 11.32), respectively.

A representative PLCγ2 phosphotyrosine immunoblot is shown in Fig. 5A. Collagen-stimulated tyrosine phosphorylation of PLCγ2 was inhibited, 30 and 120 min after ingestion of 150 mg and 300 mg of Q-4-G drink. Figure 5B shows densitometry analysis of PLCγ2 tyrosine phosphorylation for all six subjects compared with baseline (0 time point) and corrected for control. There was no significant effect of 150 mg and 300 mg Q-4-G drink ingestion on tyrosine phosphorylation of PLCγ2 30 min after ingestion. However, 120 min after ingestion of both the 150-mg and 300-mg Q-4-G drinks, tyrosine phosphorylation of PLCγ2 was inhibited significantly by 45% (SEM ± 10.3) and 38% (SEM ± 6.5), respectively ($P = 0.05$).

Discussion

The inhibitory effect of dietary flavonoids on platelet function has been recognized for some time. However, 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 plasma levels at which effects on the function of platelets may be observed. We have recently reported the identity of specific targets of collagen-mediated signaling pathways leading to platelet activation, which are inhibited by quercetin *in vitro*, and these include src-family kinases, the tyrosine kinase Syk and PI3-K [15].

Hertog *et al.* [10,19] reported an average daily intake of 30 mg of quercetin per day from dietary records, but recent reports demonstrate that the systemic availability of quercetin is highly dependent on the form of quercetin that is ingested. Q-4-G has been reported to be preferentially absorbed into the body from the gut in comparison with other forms of quercetin such as quercetin 3-O-β-rutinoside [13,20], with the highest peak plasma quercetin concentration reported in the literature as 3.5 μM. In experiments previously described [15], quercetin inhibited collagen-stimulated cell signaling and platelet aggregation *in vitro* at similar concentrations.

The aims of this pilot study were to investigate the systemic availability of a quercetin supplement, and to determine whether the levels adsorbed into plasma reach concentrations that are able to inhibit collagen-mediated signaling in platelets and thereby reduce platelet aggregation responses.

Following ingestion of the Q-4-G-containing drinks, analysis of the quercetin concentration in plasma revealed that quercetin was absorbed from the gut into the bloodstream. Plasma quercetin concentrations peaked at 4.7 μM and 9.7 μM, 30 min after ingestion of the drink for the 150-mg and 300-mg doses, respectively. Notably, these concentrations were higher than have been reported previously. In the present study, the plasma

samples were enzymically treated with a mixture of glucuronidase/sulphatase to yield free quercetin, allowing no differentiation between different conjugates such as glucuronides or sulphates which are difficult to identify in plasma, due to the lack of available standards. Thus, the concentration of quercetin measured in plasma samples encompassed all forms of quercetin (total quercetin). Without enzymic treatment, however, neither quercetin nor its methylated metabolites, isorhamnetin and tamarixetin, could be detected in plasma samples (results not shown), indicating that the aglyca do not circulate in blood after oral ingestion of Q-4-G. The plasma concentrations of isorhamnetin and tamarixetin were approximately 10-fold lower than those of quercetin. The parallel absorption and elimination profiles of all three compounds detected suggest that the methylated metabolites isorhamnetin and tamarixetin are derived from intestinal and/or hepatic metabolism of quercetin, as suggested by Manach *et al*. [21] and Ader *et al*. [22]. Total flavonoid concentrations peaked at 5.1 μM and 10.7 μM for the 150-mg and 300-mg doses, respectively, 30 min after ingestion of the drink. The conjugates of quercetin could not be analysed in plasma, therefore the identity of the dominant or bioactive conjugate form of quercetin was not addressed in this study, although given the functional effects of Q-4-G ingestion on platelet function observed in this study, this will be an important issue to resolve.

Collagen-stimulated platelet aggregation was inhibited by ingestion of both 150 mg and 300 mg Q-4-G. The inhibitory effect was dependent on the concentration of collagen used to stimulate platelet aggregation and the dose of Q-4-G ingested, consistent with previous *in vitro* data [15]. The inhibition of platelet aggregation by collagen after ingestion of 150 mg Q-4-G was time-dependent, with more potent effects seen 120 min after ingestion. This trend was apparent with all the concentrations of collagen used. With the lower concentrations of collagen, ingestion of the 300-mg dose of Q-4-G yielded potent inhibitory effects on platelet aggregation, 30 min after ingestion of the drink, which was sustained 120 min after ingestion. This indicates a maximal inhibitory effect 30 min after ingestion of the 300-mg dose of Q-4-G, in comparison with a maximal inhibitory effect 120 min after ingestion of the 150-mg dose of Q-4-G. A time-dependent inhibitory trend was seen after ingestion of the 300-mg dose of Q-4-G when platelet aggregation was stimulated with the higher concentrations of collagen. After ingestion of the 300-mg dose of Q-4-G collagen-induced platelet aggregation was not inhibited to the same degree as after ingestion of the 150-mg dose of Q-4-G. However, the sum of the 30-min and 120-min inhibition levels after ingestion of the 300-mg dose was greater than the inhibition levels after ingestion of the 150-mg dose, indicating that the inhibition occurs over different time scales.

The inhibition of platelet aggregation upon ingestion of Q-4-G was dependent on the concentration of collagen used. Therefore, although the GPVI activation pathway is inhibited by quercetin, it is probable that residual activity is present in platelets that can overcome this inhibitory effect, when high concentrations of agonist are used. This was also apparent in initial *in vitro* studies, where the activity of GPVI pathway signaling molecules including Syk, Fyn, Lyn and PI3-K was inhibited but not blocked completely [15].

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 reported little effect [23,24]. Chronic ingestion of polyphenolic-rich foods has been observed to inhibit platelet aggregation by Keevil *et al*. [25], who supplemented 10 subjects with purple grape juice for 7 days, which inhibited whole blood platelet aggregation induced with collagen (1 μg mL^{-1}) by 77%. However, other similar studies have reported no significant inhibitory effect of the ingestion of polyphenolic-rich foods on platelet aggregation [26,27]. A number of these studies used relatively high concentrations of agonist to stimulate platelet aggregation, which may have masked any inhibitory activity. In the current study the inhibition of platelet aggregation by ingestion of Q-4-G was more potent where lower concentrations of collagen were used (0.5 μg mL^{-1} and 1.0 μg mL^{-1}).

In the present study whole platelet tyrosine phosphorylation was inhibited significantly, in a time-dependent manner, after the ingestion of both the 150-mg and the 300-mg doses of Q-4-G. Of particular importance is the observation that the tyrosine phosphorylation of Syk and phospholipase Cγ2 on stimulation with collagen was inhibited significantly by ingestion of 150 mg and 300 mg Q-4-G. However, the two doses of Q-4-G (150 mg and 300 mg) had no differential effects on cell signaling events in platelets, which may indicate maximal inhibitory activity at least at the level of tyrosine phosphorylation of these specific proteins, after ingestion of 150 mg Q-4-G. The inhibitory effects of Q-4-G on platelet aggregation and signaling support previous *in vitro* work by the authors [15] and indicate that quercetin obtained in the diet is capable of reducing GPVI signaling 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 [15]. A delay is observed between maximal levels of plasma quercetin at 30 min and peak levels of inhibitory activation on platelet signaling and aggregation. It is possible that this reflects platelet membrane permeability of the active metabolite, thereby delaying the development of suitably high intracellular concentrations.

This investigation was initially undertaken as a small pilot supplementation study with six subjects. However, statistically significant inhibition of platelet function and signaling was observed after quercetin supplementation, suggesting that quercetin is a very potent bioactive molecule present in the human diet.

The doses of quercetin used in the present study are higher than is thought to be achievable from normal dietary intake; however, it has been suggested that 30 mg of quercetin can be ingested from an average diet [10,19]. We have recently found that approximately 74 mg of quercetin can be ingested from a large bowl of onion soup (equivalent to approximately three

medium-sized onions), producing plasma quercetin levels as high as 4.65 μM in one subject, which is similar to the plasma levels found in the current study (Hubbard, Wolffram, Lovegrove and Gibbins, unpublished results 2004). This suggests that quercetin could be a physiologically relevant inhibitor of platelet function *in vivo* obtained from dietary sources.

The search for compounds that produce mild inhibitory effects to prevent platelet activation has included the investigation of natural compounds that are able to inhibit platelet function, especially those found in the diet. 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 cardiovascular disease may be linked. The evidence presented here suggests that the flavonoids, and in particular quercetin, may play an important role in health and disease not only regarding the inhibition of platelet function and hence a possible decreased risk of thrombosis, but also the potential role of quercetin as an inhibitor of a number of cell-signaling mechanisms *in vivo*. The discovery that the effects of quercetin on cell-signaling mechanisms *in vitro* can be reproduced after ingestion of quercetin as a human dietary supplement may have important 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, wine and tea, which may contain high concentrations of quercetin, have a reduced cardiovascular disease risk [19].

Given the dramatic effects of Q-4-G supplementation on platelet signaling and functional responses, it will be important to identify the specific active metabolite that is responsible for the observed effects. This will enable a more detailed mode of action at the molecular level to be determined, and the therapeutic potential of quercetin supplementation to be assessed.

Acknowledgements

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