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ORIGINAL ARTICLE

# Quercetin inhibits collagen-stimulated platelet activation through inhibition of multiple components of the glycoprotein VI signaling pathway

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**Summary.** *Background:* The regulation of platelet function by pharmacological agents that modulate platelet signaling has proven a successful approach to the prevention of thrombosis. A variety of molecules present in the diet have been shown to inhibit platelet activation, including the antioxidant quercetin. *Objectives:* In this report we investigate the molecular mechanisms through which quercetin inhibits collagen-stimulated platelet aggregation. *Methods:* The effect of quercetin on platelet aggregation, intracellular calcium release, whole cell tyrosine phosphorylation and intracellular signaling events including tyrosine phosphorylation and kinase activity of proteins involved in the collagen-stimulated glycoprotein (GP) signaling pathway were investigated. *Results:* We report that quercetin inhibits collagen-stimulated whole cell protein tyrosine phosphorylation and intracellular mobilization of calcium, in a concentration-dependent manner. Quercetin was also found to inhibit various events in signaling generated by the collagen receptor GPVI. This includes collagen-stimulated tyrosine phosphorylation of the Fc receptor  $\gamma$ -chain, Syk, LAT and phospholipase  $C\gamma 2$ . Inhibition of phosphorylation of the Fc receptor  $\gamma$ -chain suggests that quercetin inhibits early signaling events following stimulation of platelets with collagen. The activity of the kinases that phosphorylate the Fc receptor  $\gamma$ -chain, Fyn and Lyn, as well as the tyrosine kinase Syk and phosphoinositide 3-kinase was also inhibited by quercetin in a concentration-dependent manner, both in whole cells and in isolation. *Conclusions:* The present results provide a molecular basis for the inhibition by quercetin of collagen-stimulated platelet activation, through inhibition of multiple components of the GPVI signaling pathway, and may begin to explain the proposed health benefits of high quercetin intake.

**Keywords:** collagen, GPVI, platelets, quercetin.

## Introduction

Platelets play a pivotal role in both health and disease, through their central involvement in hemostasis and thrombosis. When blood vessels become damaged, subendothelial macromolecules such as collagen and thrombin, which are exposed or generated at the site of damage, stimulate platelet activation and the initiation of hemostasis. Collagen supports the adhesion of platelets to the site of injury via von Willebrand factor (VWF) as well as glycoprotein VI (GPVI) and integrin  $\alpha 2\beta 1$  [1,2]. The binding of collagen to GPVI results in receptor clustering and thereby stimulates the tyrosine phosphorylation of specific tyrosine residues within an associated transmembrane protein, the Fc receptor  $\gamma$ -chain (FcR  $\gamma$ -chain) [3,4]. This leads to the recruitment of signaling proteins such as the tyrosine kinase Syk and phosphoinositide 3-kinase (PI 3-kinase) leading to the activation of several signaling pathways [4–6]. This results in platelet shape change and spreading, and the release or secretion of proactivatory substances that activate and recruit platelets to the developing thrombus.

Platelets are recognized to play important roles in aspects of cardiovascular disease, most notably through their critical involvement in causing thrombosis. In addition, platelet accumulation in the arterial wall at sites of endothelial injury has been suggested to be a key event in the initiation and propagation of atherosclerotic lesions [7].

The modulation of platelet activity using specific pharmacological agents has proven to be a successful strategy for the prevention of thrombosis. Platelet activation may also be inhibited by a number of dietary components including alcohol [8], some dietary fats [9] and antioxidants [10]. The relationship between diet, platelet function and cardiovascular disease is not fully understood.

A dietary antioxidant that has received attention with regard to its effects on platelet function is the polyphenolic compound quercetin (3,3',4',5,7-pentahydroxyflavone) [11], found in high concentrations in onions, apples, wine and tea [11]. A high dietary intake of this compound and others of the same family has been correlated with a decreased risk of coronary heart disease [12]. This is believed to be mediated through a wide spectrum of pharmacological effects. Quercetin has been shown to be able to inhibit low-density lipoprotein oxidation *in vitro* [13] and cause vasodilation in rat aortic strips [14]. Further-

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more, quercetin has been shown *in vitro* to inhibit platelet aggregation [15–17]. However, a mechanism for this action has not been elucidated. In this study we have investigated the effects of quercetin on collagen-stimulated platelet activation and signaling. We show that quercetin inhibits the activity of a number of critical molecules involved in the GPVI collagen receptor-stimulated signaling pathway in platelets.

## Experimental procedures

### Materials

Quercetin dihydrate was solubilized in dimethylsulfoxide (DMSO) both purchased from Sigma (Poole, UK). Horm-Chemie collagen (collagen-fibres from equine tendons) was purchased from Nycomed (Munich, Germany). Anti-phosphotyrosine monoclonal antibody (4G10) and anti-p85 phosphoinositide 3-kinase were from Upstate Biotechnology (TCS Biologicals, Botolph Claydon, Bucks, UK). Anti-Syk (N-19), anti-Syk (LR), anti-LAT, anti-PLC $\gamma$ 2, anti-Fyn and anti-Lyn polyclonal antibodies were purchased from Santa Cruz Biotechnology (Autogen Bioclear UK Ltd, Calne, Wilts, UK), and anti-Lyn (clone 42) monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies, the enhanced chemiluminescence detection system and the  $\gamma$ -<sup>32</sup>P-ATP were purchased from Amersham Biosciences Biotech (Little Chalfont, UK). L- $\alpha$ -phosphoinositide and bovine thrombin were from Sigma. Fura-2 AM was from Molecular Probes (Cambridge Bioscience, Cambridge, UK). All other reagents were from previously described sources [18,19].

### Preparation and stimulation of platelets

Human platelets from healthy aspirin-free volunteers were prepared on the day of the experiment by differential centrifugation as described previously [5] and suspended in modified Tyrodes-HEPES buffer (134 mM NaCl, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.9 mM KCl, 12 mM NaHCO<sub>3</sub>, 20 mM HEPES, 5 mM glucose, 1 mM MgCl<sub>2</sub>, pH 7.3) to a density of  $2 \times 10^8$  cells mL<sup>-1</sup> for aggregation experiments. For protein precipitation experiments, platelets were suspended at  $8 \times 10^8$  cells mL<sup>-1</sup> in buffer containing 1 mM EGTA to prevent aggregation. Stimulation of platelets (450  $\mu$ L) with collagen (50  $\mu$ L) was performed at 37 °C in an optical platelet aggregometer (Chrono-log Corp., Havertown, PA, USA) with continuous stirring (1200 r.p.m.). Platelets were incubated with quercetin dissolved in DMSO (1  $\mu$ L) or with DMSO alone (1  $\mu$ L) (0.2% v/v) for 5 min (after 10 s stirring) followed by stimulation with collagen for 90 s. No changes in pH after quercetin or collagen treatment were detected in platelet suspensions.

### Immunoprecipitation studies

Platelet stimulation was terminated by the addition of an equal volume of ice-cold NP40 lysis buffer [2% (v/v) Nonidet P40,

20 mM Tris, 300 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 10  $\mu$ g mL<sup>-1</sup> leupeptin, 10  $\mu$ g mL<sup>-1</sup> aprotinin, 1  $\mu$ g mL<sup>-1</sup> pepstatin A, pH 7.3]. Detergent-insoluble debris was removed and the lysates were pre-cleared by mixing with protein A-Sepharose (PAS) for 1 h at 4 °C (60  $\mu$ L of a 50% (w/v) suspension in Tris-buffered saline–Tween [TBS–T; 20 mM Tris, 137 mM NaCl, 0.1% (v/v) Tween 20, pH 7.6]. PAS was removed from the lysates before the addition of antibody (1  $\mu$ g mL<sup>-1</sup>). Following rotation for 1 h at 4 °C, 60  $\mu$ L PAS suspension were added to each sample and rotated for a further 1 h at 4 °C before washing the Sepharose pellet in lysis buffer and the addition of Laemmli sample-treatment buffer. Proteins were separated by SDS–PAGE under reducing conditions using 10% gels and transferred to polyvinylidene difluoride (PVDF) membranes by semidry Western blotting.

### Fusion protein precipitation studies

A GST fusion protein containing the tandem SH2 domains of Syk (GST-SYK-SH2) was expressed and purified as described elsewhere [20], and bound to glutathione-agarose. Precipitation of platelet proteins with the fusion construct was performed as described previously [3]. Precipitated proteins were separated by SDS–PAGE under reducing conditions, and transferred to polyvinylidene difluoride membranes.

### Immunoblotting

Membranes were blocked by incubation in 10% (w/v) bovine serum albumin (BSA) dissolved in TBS–T. Primary and secondary antibodies were diluted in TBS–T containing 2% (w/v) BSA, and incubated with membranes for 1 h at room temperature with rotation. Blots were washed for 2 h in TBS–T following each incubation with antibodies and then developed using an enhanced chemiluminescence detection system. Primary antibodies were used at a concentration of 1  $\mu$ g mL<sup>-1</sup> [antiphosphotyrosine 4G10; anti-Syk (N-19); anti-Fyn; anti-Lyn (clone 42); anti-PLC $\gamma$ 2; anti-LAT; anti-p85 phosphatidylinositol 3-kinase] and horseradish peroxidase (HRP)-conjugated secondary antibodies (antirabbit-IgG-HRP; antimouse-IgG-HRP) were diluted 1 : 10000.

### Measurement of [Ca<sup>2+</sup>]<sub>i</sub> by spectrofluorimetry

Washed human platelets (as above) were incubated at  $2 \times 10^9$  cells mL<sup>-1</sup> in calcium-free Tyrodes-HEPES buffer with 3  $\mu$ M Fura-2 AM for 45 min. Platelets were washed once and resuspended at  $2 \times 10^8$  cells mL<sup>-1</sup> in modified Tyrodes-HEPES buffer. Platelets (450  $\mu$ L) were incubated with quercetin dissolved in DMSO (1  $\mu$ L) or DMSO alone (0.2% v/v) for 5 min and then stimulated with collagen (50  $\mu$ L) with constant stirring at 37 °C 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/

380 nm) was calculated and converted to calcium concentration using FLWinLab software (Perkin Elmer) using the equation  $[Ca^{2+}]_i = K_d \times (R - R_{min}) / (R_{max} - R) \times SFB$  [where R is emission ratio value (340/380 nm)].  $R_{max}$ , the maximum 340/380 ratio, was determined by lysing platelets with NP40 immunoprecipitation lysis buffer in the presence of 1 mM  $CaCl_2$ . The  $R_{min}$  340/380 nm ratio was determined by addition of 2 mM EGTA.  $K_d$  is the dissociation constant of the Fura-2/ $Ca^{2+}$  complex (224 nM) and SFB is the fluorescence ratio at 340/380 nm of  $R_{min}$  and  $R_{max}$ .

#### Assay of in vitro kinase activity

Syk, Fyn or Lyn were immunoprecipitated and assayed for kinase activity whilst immobilized on protein-A Sepharose following the final wash step. Immunoprecipitates were suspended in assay buffer (105 mM NaCl, 20 mM HEPES pH 7.4, 5 mM  $MnCl_2$ , 5 mM  $MgCl_2$ , 2 mM NaF, 1 mM  $Na_3VO_4$ , 10  $\mu$ M ATP together with 5  $\mu$ Ci of  $\gamma$ - $^{32}P$ -ATP per reaction) and incubated at 30 °C for 20 min prior to termination by the addition of Laemmli SDS-PAGE sample treatment buffer. Proteins were separated by SDS-PAGE, transferred to PVDF membranes and exposed to storage phosphor screens to detect the incorporation of  $^{32}P$  into proteins present in the immunoprecipitated kinase (i.e. autophosphorylation). In some assays platelets were not incubated with quercetin or DMSO prior to stimulation. Quercetin or DMSO was instead added to the assay mixture prior to addition to the immunoprecipitate to investigate potential direct effects of quercetin on enzyme activity.

#### Phosphoinositide 3-kinase assay

Phosphoinositide 3-kinase (PI 3-kinase) was immunoprecipitated from platelets using an anti-p85 (regulatory subunit) antibody, and assayed for kinase activity while immobilized on protein-A Sepharose. PI 3-kinase activity was measured as described elsewhere [21]. Briefly, the immune complex pellet was washed twice in kinase buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 1 mM DTT), resuspended in kinase buffer with L- $\alpha$ -phosphoinositide vesicles (200  $\mu$ g  $mL^{-1}$  in 5 mM HEPES pH 7.4) and incubated for 20 min at 4 °C. The enzyme reaction was initiated by adding ATP-mix (5 mM  $MgCl_2$ , 100  $\mu$ M ATP together with 1  $\mu$ Ci  $\gamma$ - $^{32}P$ -ATP per reaction). The reaction was incubated for 10 min at 30 °C and terminated by addition of 1 M HCl. Lipids were extracted and separated by thin layer chromatography (TLC). Products were visualized by exposing TLC plates to phosphor screens for 2–6 h. The reaction products were quantified using a phosphorimager with ImageQuant software (Amersham Biosciences). In some assays platelets were not incubated with quercetin or DMSO prior to stimulation. Quercetin or DMSO was instead added to the assay mixture prior to addition to the immunoprecipitate.

#### Statistical analysis

$IC_{50}$  values were calculated using Prism for Windows version 2.0 (Graphpad Inc., San Diego, CA, USA) using non-linear

regression. Graphs were plotted with 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 standard error of mean and *P*-value  $\leq 0.05$  considered significant.

## Results

### Quercetin inhibits collagen-stimulated platelet aggregation

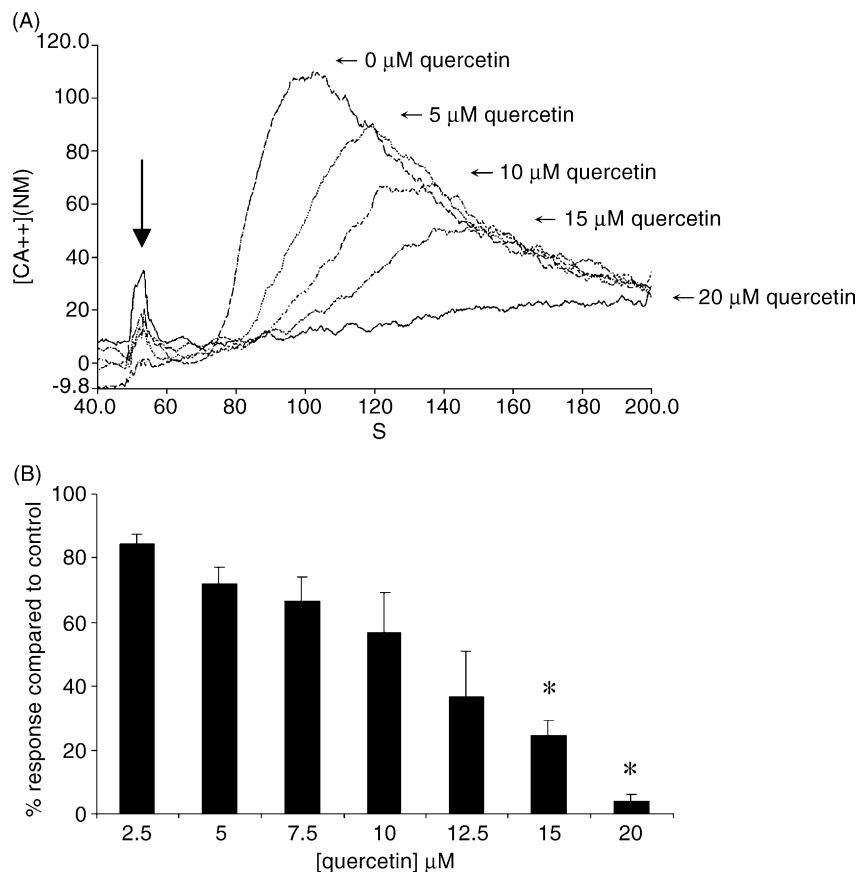
Quercetin has been shown previously to inhibit platelet aggregation in both washed platelets and platelet-rich plasma, with a number of different agonists including collagen, thrombin, ADP and arachidonic acid. Beretz *et al.* (1982) [15] used collagen as an agonist at 2.5  $\mu$ g  $mL^{-1}$  and reported an  $IC_{50}$  for quercetin of 55  $\mu$ M. However, Landolfi *et al.* (1984) [16] stimulated platelets with arachidonic acid (150  $\mu$ M) and reported an  $IC_{50}$  for quercetin of 18  $\mu$ M. It was necessary to confirm this inhibitory action in our experimental system.

Platelets were incubated with quercetin or solvent alone for 5 min and then stimulated with collagen for 90 s. Quercetin was observed to cause a concentration-dependent inhibition of platelet aggregation (not shown). In Table 1  $IC_{50}$  values for quercetin-mediated inhibition of platelet aggregation at different concentrations of collagen (0.5, 1, 2, 3, 4 and 5  $\mu$ g  $mL^{-1}$ ) are given. Results are derived from three separate experiments. DMSO (0.2% v/v) was used as solvent control and had no effect on platelet aggregation at this concentration. The  $IC_{50}$  values obtained were dependent on the concentration of collagen used, where lower concentrations of quercetin were required to cause 50% inhibition of platelet aggregation at lower concentrations of collagen. The  $IC_{50}$  value for the highest concentration of collagen used (5  $\mu$ g  $mL^{-1}$ ), is lower than previously reported ( $IC_{50}$ : 8.7  $\mu$ M) [15,16]. Quercetin also inhibited platelet aggregation in response to other agonists. However, this effect was less potent in comparison with the inhibitory effect of quercetin on collagen-stimulated platelet aggregation with notably higher  $IC_{50}$  values, i.e. thrombin 0.1 IU  $mL^{-1}$ ,  $IC_{50}$  27.6  $\mu$ M ( $\pm 1.04$ ).

**Table 1**  $IC_{50}$  values for the inhibition of platelet aggregation by quercetin with varying concentrations of collagen

Collagen $\mu$ g $mL^{-1}$	$IC_{50}$ (quercetin) $\mu$ M ( $n = 3$ ) $\pm$ SEM
5	8.69 $\pm$ 1.05
4	6.47 $\pm$ 1.09
3	4.93 $\pm$ 1.12
2	4.87 $\pm$ 1.11
1	3.02 $\pm$ 1.05
0.5	2.37 $\pm$ 1.03

Washed platelets were incubated with quercetin or solvent control for 5 min and then stimulated with collagen for 90 s. The inhibition of platelet aggregation was determined in comparison with control and  $IC_{50}$  values were calculated.



**Fig. 1.** Quercetin inhibits collagen-stimulated mobilization of calcium from intracellular stores in a concentration-dependent manner. Fura-2 AM-loaded platelets were incubated with quercetin or solvent control for 5 min and then stimulated with collagen ( $5 \mu\text{g mL}^{-1}$ ) for 200 s. The mobilization of calcium was measured by spectrofluorimetry as described in Methods. (A) A representative set of calcium responses (calcium concentration  $[CA^{2+}]$  (nM) vs. time [s]) following preincubation with solvent control (DMSO) or quercetin (5–20  $\mu\text{M}$ ) (arrow indicates the addition of agonist). (B) Mean peak calcium concentration data shown as a percentage response compared with control for a range of quercetin concentrations ( $n = 3$ ,  $\pm$  SEM,  $*P \leq 0.005$ ).

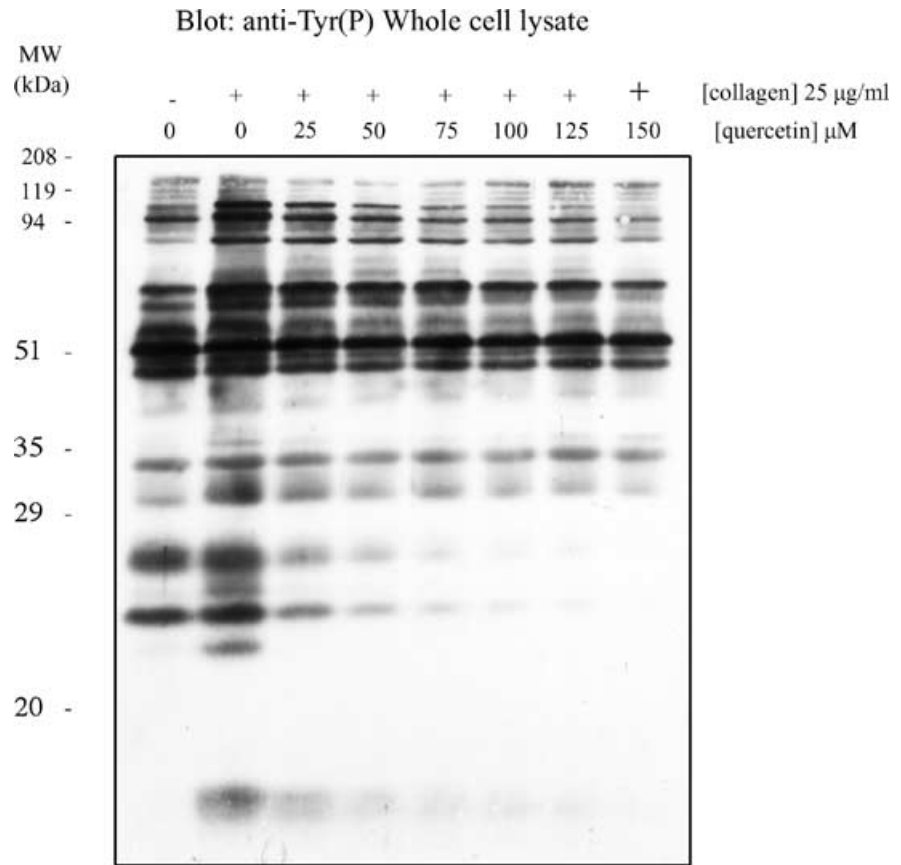
#### *Quercetin inhibits collagen-stimulated mobilization of calcium from intracellular stores in a concentration-dependent manner*

Stimulation of the collagen receptor GPVI leads to rapid intracellular mobilization of calcium, which is essential for platelet secretion and aggregation [22]. To investigate the effect of quercetin on the intracellular mobilization of calcium stores, calcium levels were measured fluorometrically using the calcium-sensitive dye, Fura-2 AM. Experiments were performed in the presence of 2 mM EGTA to prevent the influx of extracellular calcium. Fura-2 AM-loaded platelets were preincubated with quercetin or solvent alone for 5 min and then stimulated with collagen ( $5 \mu\text{g mL}^{-1}$ ). Stimulation of platelets with collagen resulted in a rapid increase in intracellular calcium concentration (Fig. 1A). Quercetin caused a concentration-dependent decrease in collagen-stimulated intracellular calcium release. This inhibitory response is within the same concentration range as that shown in Table 1 ( $IC_{50} = 9.4 \mu\text{M}$ ) for aggregation at this concentration of collagen. Mean peak calcium concentration from separate experiments ( $n = 3$ ,  $\pm$  SEM) as a percentage of maximal response is shown in Fig. 1B.

#### *Collagen-stimulated platelet protein tyrosine phosphorylation is inhibited by quercetin*

Given the marked effect of quercetin on platelet aggregation, we investigated the effect of quercetin on collagen-stimulated

signal transduction. Stimulation of platelets with collagen results in the activation of a tyrosine kinase-dependent signaling pathway [3,4,6]. Consequently, platelet activation with collagen is accompanied by tyrosine phosphorylation of multiple platelet proteins. The effect of quercetin on levels of protein tyrosine phosphorylation was examined in platelet whole cell lysates (Fig. 2). Consistent with previous reports [23] collagen ( $25 \mu\text{g mL}^{-1}$ ) induced an increase in the protein tyrosine phosphorylation of a number of platelet proteins in comparison with basal levels. Pretreatment of platelets with quercetin for 5 min prior to stimulation with collagen ( $25 \mu\text{g mL}^{-1}$ ) resulted in a concentration-dependent inhibition of protein tyrosine phosphorylation. In these and the following experiments, platelets were stimulated under non-aggregating conditions due to the inclusion of EGTA (1 mM), which chelates calcium, in order to ensure the study of primary signaling events via collagen, rather than secondary stimulation following secretion and aggregation. This standard approach to study platelet signaling, also holds the advantage that aggregation-dependent relocation of signaling proteins to insoluble cytoskeletal fractions is prevented. This facilitates immunoprecipitation of equivalent levels of signaling proteins from resting and activated platelets, which is essential for the experiments described below. Due to the reduced levels of positive feedback signaling as a result of EGTA treatment it is necessary to increase collagen concentrations to  $25 \mu\text{g mL}^{-1}$  in order to observe a substantial increase in protein tyrosine phosphorylation. Quercetin concentrations used were increased proportionately (5-fold) when compared



**Fig. 2.** Collagen-stimulated platelet protein tyrosine phosphorylation is inhibited by quercetin. Platelets in the presence of EGTA (1 mM) were incubated with quercetin or solvent control for 5 min and then stimulated with collagen ( $25 \mu\text{g mL}^{-1}$ ) for 90 s. Platelets were lysed and proteins separated by SDS-PAGE under reducing conditions and immunoblotted to detect protein phosphotyrosine residues. Data are representative of three separate experiments.

with the concentrations used in Fig. 1. The addition of relatively large volumes of collagen in acid buffer did not exceed the buffering capacity of the modified Tyrodes buffer and the pH remained at 7.3.

#### *Quercetin inhibits the tyrosine phosphorylation of multiple components of the GPVI signaling pathway*

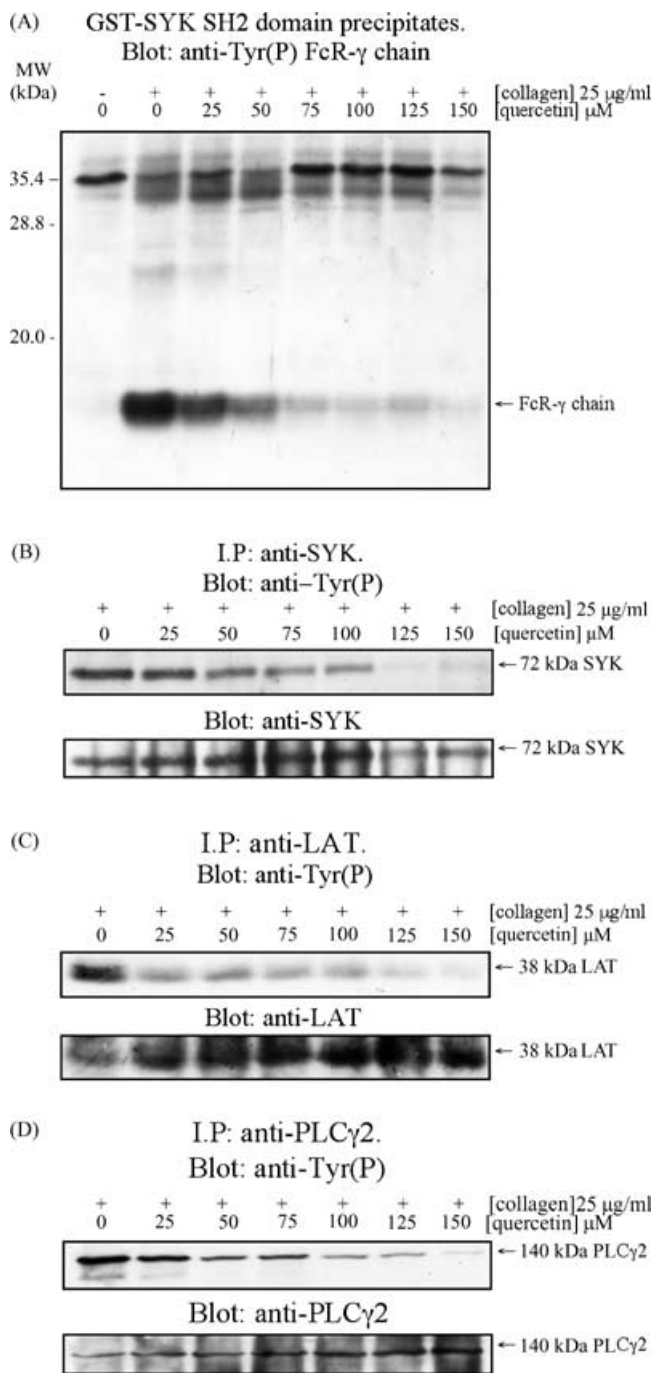
To examine the mechanism through which quercetin inhibits collagen receptor-mediated signaling we examined the effect of this compound on the tyrosine phosphorylation of a number of components of the collagen-signaling pathway. Activation of the GPVI collagen receptor results in tyrosine phosphorylation of the associated receptor-complex protein, the FcR  $\gamma$ -chain [3,4]. The effect of quercetin on the level of tyrosine phosphorylation of this protein following stimulation with collagen was therefore examined. Quercetin caused the level of collagen-stimulated tyrosine phosphorylation of the FcR  $\gamma$ -chain to become diminished in a concentration-dependent manner (Fig. 3A). A substantial decrease in tyrosine phosphorylation of the FcR  $\gamma$ -chain was observed on treatment with  $25 \mu\text{M}$  quercetin, and was completely inhibited at a concentration of  $150 \mu\text{M}$ . This indicates that quercetin is able to inhibit very early signaling events in the collagen signaling pathway. To confirm this, molecules that had been shown to lie downstream of the FcR  $\gamma$ -chain were also examined. As may be anticipated, collagen-stimulated tyrosine phosphorylation of the non-recep-

tor tyrosine kinase Syk was also inhibited by quercetin in a concentration-dependent manner (Fig. 3B) with a similar profile to that of the FcR  $\gamma$ -chain. Activation of Syk following its association with the activated receptor complex results in tyrosine phosphorylation of the transmembrane adapter protein LAT [5], which in turn leads to the recruitment of phospholipase  $\text{C}\gamma 2$  (PLC $\gamma 2$ ) [24]. We therefore also investigated the effect of quercetin on collagen-stimulated tyrosine phosphorylation of LAT (Fig. 3C) and PLC $\gamma 2$  (Fig. 3D). The levels of tyrosine phosphorylation of LAT and PLC $\gamma 2$  were also inhibited in a concentration-dependent manner by quercetin.

Collagen-stimulated platelet activation via GPVI is therefore inhibited by quercetin, but it was unclear whether quercetin exerts effects at the top of the pathway alone or whether it has effects at other locations in the GPVI signaling pathway. Reports of the inhibitory effects of quercetin or related compounds on a number of signaling molecules indicated that this was a possibility [25]. The effect of quercetin on the enzymatic activities of specific components of the pathways activated by GPVI were therefore examined.

#### *The kinase activities of the Src-family kinases Fyn and Lyn and the tyrosine kinase Syk are inhibited by quercetin*

The tyrosine phosphorylation of the FcR  $\gamma$ -chain following collagen binding to GPVI is mediated by the Src-family kinases Fyn and Lyn [26,27]. This leads to the recruitment and activa-



**Fig. 3.** Quercetin inhibits the tyrosine phosphorylation of multiple components of the GPVI signaling pathway. Platelets in the presence of EGTA (1 mM) were incubated with quercetin or solvent control for 5 min and then stimulated with collagen (25  $\mu$ g mL $^{-1}$ ) for 90 s. (A) Platelet cell lysates were incubated with GST-Syk tandem SH2 domains fusion protein for 2 h at 4  $^{\circ}$ C. Precipitated proteins were separated by SDS-PAGE and immunoblotted to detect protein phosphotyrosine residues. The low-molecular-weight band indicated has previously been identified as the FcR  $\gamma$ -chain. (B,C,D) Platelet lysates were incubated with anti-Syk (LR), anti-LAT or anti-PLC $\gamma$ 2, respectively, and protein A-Sepharose (PAS) for 2 h at 4  $^{\circ}$ C. Precipitated proteins were separated by SDS-PAGE and immunoblotted to detect phosphotyrosine residues. Equivalent protein loading was verified by reprobing for Syk, LAT or PLC $\gamma$ 2. Data are representative of three separate experiments.

tion of the cytosolic tyrosine kinase Syk. These three proteins were investigated for an inhibitory effect of quercetin on their kinase activities (Fig. 4). Fyn and Lyn were immunoprecipitated from platelets stimulated with collagen (25  $\mu$ g mL $^{-1}$ ) in the presence and absence of quercetin and assayed for kinase activity *in vitro*. As shown in Fig. 4Ai,Bi, the kinase activities of Fyn and Lyn, respectively, were inhibited by quercetin in a concentration-dependent manner. Quercetin was also seen to inhibit the kinase activity of Syk (Fig. 4Ci) in a concentration-dependent manner.

Since Fyn and Lyn are involved in initiating GPVI signaling, our results suggest that quercetin may directly inhibit the activity of Fyn and Lyn. We therefore examined the ability of quercetin directly to inhibit these enzymes. This was achieved by incubating immunoprecipitated Fyn or Lyn kinase with quercetin before measuring kinase activity *in vitro*. The kinase activities of Fyn and Lyn were seen to be inhibited directly by quercetin in a concentration-dependent manner (Fig. 4Aii,Bii, respectively). As is common with kinase inhibitors, the direct inhibitory action of quercetin was more potent than its effect on whole cells. The potential ability of quercetin to inhibit Syk kinase activity directly was also examined in the same way as for Fyn and Lyn. Quercetin also directly inhibited Syk (Fig. 4Cii) in a concentration-dependent manner.

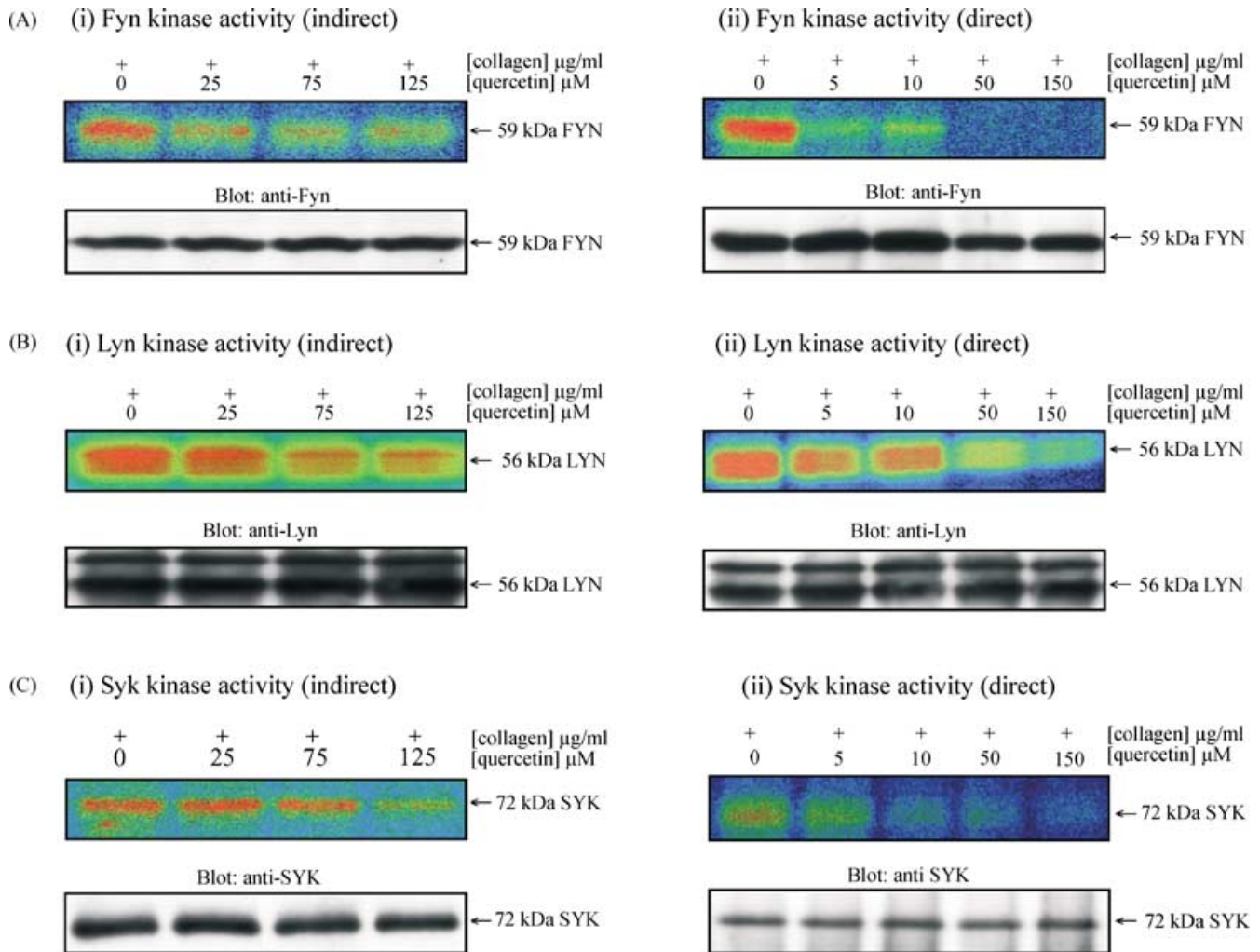
#### Quercetin inhibits collagen-stimulated PI 3-kinase activity

PI 3-kinase plays a critical role in the activation of platelets and is recruited to the phosphorylated FcR  $\gamma$ -chain and LAT upon activation of GPVI [5]. The effect of quercetin on PI 3-kinase activity was therefore investigated in a similar format to that of the other kinases (see above). Platelets were preincubated with quercetin or solvent alone and then stimulated with collagen (25  $\mu$ g mL $^{-1}$ ). PI 3-kinase was immunoprecipitated from platelet lysates and kinase activity was assayed *in vitro* using phosphoinositide as substrate. As shown in Fig. 5A, quercetin inhibited significantly collagen-induced PI 3-kinase activity in a concentration dependent manner ( $*P \leq 0.005$  for 125  $\mu$ M quercetin). In a second series of experiments, the ability of quercetin directly to inhibit PI 3-kinase isolated from platelets was examined. Platelets were stimulated with collagen (25  $\mu$ g mL $^{-1}$ ) before isolation of PI 3-kinase and determination of kinase activity in the presence or absence of quercetin (Fig. 5B). In these experiments quercetin significantly inhibited PI 3-kinase activity directly ( $*P \leq 0.005$  with 10, 50 and 150  $\mu$ M quercetin) and with a higher potency than seen with preincubation of platelets with quercetin (Fig. 5A).

#### Discussion

A group of antioxidants of the flavonoid family has been shown to inhibit platelet aggregation *in vitro* [15–17] and human studies have also suggested that a diet high in flavonoids may inhibit platelet aggregation *in vivo* [28]. Quercetin, a





**Fig. 4.** The kinase activities of the Src-family kinases Fyn and Lyn, and the tyrosine kinase Syk are inhibited by quercetin. For experiments investigating the effect of quercetin on kinase activity via the GPVI collagen activation pathway (i), washed platelets in the presence of EGTA (1 mM) were incubated with quercetin or solvent control for 5 min and then stimulated with collagen ( $25 \mu\text{g mL}^{-1}$ ) for 90 s. Platelet cell lysates were then incubated with anti-Fyn (A), anti-Lyn (B), or anti-Syk(LR) (C), and protein A-Sepharose (PAS) for 2 h at  $4^\circ\text{C}$ . Immunoprecipitates were assayed for kinase activity as described in Methods. For experiments investigating the direct effect of quercetin on kinase activity (ii), immunoprecipitates (as above) were resuspended in kinase buffer with quercetin or solvent control and then assayed for kinase activity. For both experiments incorporation of  $^{32}\text{P}$  into the immunoprecipitated protein was detected by phosphorimager. Immunoprecipitation was verified by reprobing for the appropriate antibody to ensure equal loading. Data are representative of three separate experiments.

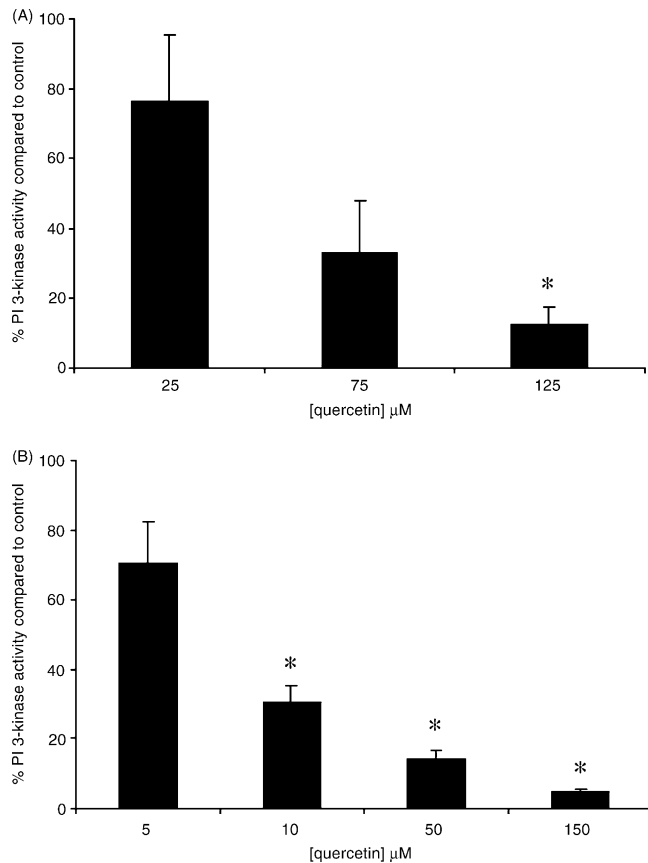
flavonoid that has received much attention in the area of nutritional biology, has been shown to inhibit platelet aggregation *in vitro* [15], although a specific mechanism by which this inhibition occurs has not been established. Reports have indicated that a number of intracellular events associated with the control of platelet activation and hemostasis are also affected following treatment of platelets with quercetin. These include inhibition of the generation of metabolites of arachidonic acid by cyclooxygenase and lipoxygenase [16,29], and inhibition of calcium-dependent isoforms of protein kinase C (PKC) [30].

Consistent with previous reports, quercetin was shown to inhibit collagen-induced platelet aggregation *in vitro* in a concentration-dependent manner. This was also dependent on the concentration of collagen used, with higher concentrations of quercetin being required to overcome stimulation by higher

concentrations of collagen. All the  $\text{IC}_{50}$  values for quercetin reported in this study are lower than those previously published [15,16]. Discrepancies with published  $\text{IC}_{50}$  values are likely to be a result of different experimental procedures and reagents. It is particularly interesting to note that the  $\text{IC}_{50}$  values reported here fall within or close to concentrations that may be present in the plasma. Quercetin levels in blood plasma after ingestion of certain forms of quercetin have been reported as high as  $3 \mu\text{M}$  [31]. Therefore quercetin derived from the normal diet or through supplementation may have an inhibitory effect on platelet function *in vivo*.

Due to the potent inhibitory effect of quercetin on collagen-stimulated platelet aggregation in comparison with other agonists such as thrombin, the effect of quercetin on the collagen-stimulated signaling pathway was examined. Quercetin was found to inhibit both collagen-stimulated protein





**Fig. 5.** Quercetin inhibits collagen-stimulated phosphoinositide (PI) 3-kinase activity. (A) Washed platelets in the presence of EGTA (1 mM) were preincubated with quercetin or solvent control for 5 min and then stimulated with collagen ( $25 \mu\text{g mL}^{-1}$ ) for 90 s. PI 3-kinase was immunoprecipitated from platelet lysates and resuspended in kinase buffer containing phosphoinositide vesicles and incubated for 20 min at  $4^\circ\text{C}$ .  $\gamma\text{-}^{32}\text{P}\text{-ATP}$  was then added and incubated for 10 min at  $30^\circ\text{C}$ . Lipids were extracted and separated by thin layer chromatography and incorporation of  $^{32}\text{P}$  into the lipid product phosphoinositide 3,4,5-trisphosphate was detected by phosphorimager. Data represent mean percentage  $^{32}\text{P}$  incorporation compared with control as a measure of PI 3-kinase activity ( $n = 3$ ,  $\pm \text{SEM}$ ,  $*P \leq 0.005$ ). (B) The direct effect of quercetin on PI 3-kinase activity was examined by incubating immunoprecipitates in kinase buffer containing phosphoinositide vesicles and quercetin or solvent control. Kinase activity was determined as above. Data represent mean percentage  $^{32}\text{P}$  incorporation compared with control as a measure of PI 3-kinase activity with direct inhibition by quercetin ( $n = 3$ ,  $\pm \text{SEM}$ ,  $*P \leq 0.005$ ).

tyrosine phosphorylation and collagen-stimulated calcium mobilization from intracellular stores. These processes are both essential for platelet activation and play a major role in platelet aggregation [4,22], although this may be explained by inhibition of a range of signaling proteins that lie upstream of these events.

We therefore investigated the effect of quercetin on specific molecules of the collagen-stimulated activation signaling pathway. Quercetin was found to inhibit tyrosine phosphorylation of the FcR  $\gamma$ -chain and therefore appears to have an inhibitory effect at the top of the collagen-stimulated activation pathway. Tyrosine phosphorylation of the FcR  $\gamma$ -chain is

thought to involve the activity of two Src-family kinases, namely Fyn and Lyn [26,27]. We have also shown that quercetin is able to inhibit the kinase activity of Fyn and Lyn in a concentration-dependent manner, both via GPVI (in whole cells) and directly. Consequently, quercetin is able to inhibit collagen-stimulated tyrosine phosphorylation of the FcR  $\gamma$ -chain.

Since it has been suggested that quercetin inhibits a range of signaling proteins, additional molecules further down the GPVI collagen activation pathway were examined for the effect of quercetin on their activation. Quercetin was shown to inhibit both the tyrosine phosphorylation and the kinase activity of Syk, although this is likely to be in part a consequence of the inhibition of Fyn and Lyn, and thereby the signaling downstream of these. However, we also found that quercetin has a direct inhibitory effect on Syk kinase activity. Tyrosine phosphorylation and activation of Syk is thought to lead to the phosphorylation of tyrosine residues in the transmembrane protein LAT and subsequently PLC $\gamma$ 2, and this was also inhibited by quercetin. The recruitment and binding of PI 3-kinase to tyrosine phosphorylated LAT is involved in the activation of this enzyme and generation of 3'-phosphorylated inositol phospholipid second messengers. Quercetin was therefore also suspected to have an inhibitory effect on the activation of PI 3-kinase. Indeed, the activity of PI 3-kinase was found to be inhibited by quercetin, both via GPVI (in whole cells) and directly. The direct effect of quercetin on PI 3-kinase activity is consistent with a number of studies [25,32]. It has been suggested that quercetin is ATP-competitive on PI 3-kinase [32]. This is consistent with our observations for PI 3-kinase, and also Fyn, Lyn and Syk, where the inhibitory action of quercetin is more potent when incubated directly with isolated kinases in comparison with its effect on these enzymes when incubated with intact platelets where high ATP concentrations are present. This effect may indicate a mechanism of action.

The potent ability of quercetin to inhibit platelet function, compared with other dietary components that have been studied, is likely to reflect its capacity to inhibit a wide range of signaling enzymes involved in the activation process. We have shown this to include tyrosine and lipid kinases, and evidence from the literature suggests that this compound will also inhibit serine/threonine kinases [25].

This study has concentrated on the effects of quercetin on platelet signaling by an activation pathway that is absolutely dependent on the activation of kinases. It is noteworthy that G protein-coupled thrombin receptor-mediated activation of platelets, which is less dependent on protein kinases, is less sensitive to inhibition by quercetin. The inhibitory effect of quercetin on thrombin-mediated activation is likely to be because thrombin signaling also leads to activation of PI 3-kinase. Therefore, although this dietary component appears to be a relatively non-selective inhibitor of kinases, its effect on platelet function depends on the activation pathway that is stimulated.

The activation of platelets is associated with the generation of reactive oxygen species which are believed to be involved in the

regulation of platelet responses [33]. This is possibly through the inhibition of protein phosphatases, although this is not fully characterized. In this study we have not examined the possibility that the antioxidant capacity of this molecule may play a role in its inhibitory activity.

In summary, we have established in this study that the potent inhibitory action of the dietary flavonoid quercetin on platelet function is a result of the ability of this compound to inhibit multiple components of collagen-stimulated signaling pathways in platelets. The relationship between diet and cardiovascular disease is complex, but the observations described in this study may begin to address the beneficial effects of a diet rich in flavonoids on the cardiovascular system. Further work is required to determine whether quercetin may be beneficial in the prevention of thrombosis, from diet or supplementation.

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