Quercitrin inhibits platelet activation in arterial thrombosis

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Abstract

Background and Purpose: The ingestion of flavonoids has been reported to be associated with reduced cardiovascular disease risk. Among flavonoids, quercitrin is the most common flavonoid in nature, and it exhibits anti-oxidant properties. However, it is unclear whether quercitrin plays a role in thrombogenesis. Experimental Approach: The anti-platelet effect of quercitrin was assessed using platelet aggregation, granule secretion, calcium mobilization, integrin activation, and western blot. Antithrombotic effect was determined in mouse using FeCl3-induced arterial thrombus formation in vivo and thrombus formation on collagen-coated surfaces under arteriolar shear in vitro. Transection tail bleeding time was used to evaluate adverse effects. Key Results: Quercitrin significantly impaired CRP- or U46619-induced platelet aggregation, granule secretion, ROS generation, and intracellular Ca2+ mobilization. Outside-in signaling of αIIbβ3 integrin was significantly inhibited by quercitrin in a concentration-dependent manner. The inhibitory effect of quercitrin resulted from inhibition of the GPVI- or U46619-mediated phosphorylation of PLC and PI3K signaling during platelet activation. Further, the anti-oxidant effect is derived from decreased phosphorylation of components of the TRAF4/p47phox/Hic5 axis signalosome. Oral administration of quercitrin efficiently blocked FeCl3-induced arterial thrombus formation in vivo and thrombus formation on collagen-coated surfaces under arteriolar shear in vitro, without prolonging bleeding time. Studies using a mouse model of ischemia/reperfusion-induced stroke indicated that treatment with quercitrin reduced the infarct volume in stroke. Conclusions and Implications: Our results demonstrated that quercitrin could be an effective therapeutic agent for the treatment of thrombotic diseases.

Abbreviations

CRP: collagen-related peptide; **ROS:** reactive oxygen species; **GPVI:** glycoprotein VI; **PLC:** phospholipase C; **PI3K:** phosphoinositide 3-kinase; **TRAF4:** TNF receptor-associated factor 4; **FeCl₃:** ferric chloride

What is already know

- The ingestion of flavonoids has been associated with reduced cardiovascular disease risk.
- Quercitrin has anti-oxidant properties.

What does this study add

- Quercitrin effectively reduces the platelet thrombus formation *in vivo* and *in vitro,* without inducing bleeding.
- Oral administration of quercitrin has immediate benefits in thrombus formation and a mouse model of ischemia/reperfusion-induced stroke.

What is the clinical significance

Quercitrin could be an effective therapeutic agent for the treatment of thrombotic diseases.

INTRODUCTION

The response of platelets to vascular damage is essential for hemostasis, whereas excessive platelet activation leads to thrombotic vascular conditions such as stroke, myocardial infarction, and arterial thrombosis.(Yeung, Li & Holinstat, 2018) In response to vascular injury, platelets rapidly adhere to immobilized adhesive proteins such as von Willebrand factor and collagen, thereby triggering the activation and aggregation of platelets and subsequent thrombus formation.(Furie & Furie, 2008) Collagen-induced platelet activation is initiated by binding to the primary glycoprotein VI (GPVI) receptor. The signaling pathway is initiated by Src family kinase-mediated phosphorylation of tyrosine residues associated with immunoreceptor tyrosine-based activation motif-containing FcRγ chains.(Nieswandt & Watson, 2003) Subsequently, phospholipase Cγ2 (PLCγ2) is activated by the signaling cascades involved in the recruitment and activation of spleen tyrosine kinase (Syk), lymphocyte cytosolic protein 2 (SLP-76), Vav1, phosphatidylinositol 3-kinase (PI3K), and Bruton's tyrosine kinase (Btk). The tyrosine phosphorylation-based activation of PLCγ2 eventually leads to intracellular Ca^{2+} accumulation, a marker of platelet activation and thrombus formation.(Munnix et al., 2005; Varga-Szabo, Braun & Nieswandt, 2009) In addition, TNF receptor-associated factor 4 (TRAF4), as a binding partner of p47^{phox} in NADPH oxidase 1 and 2 complexes, interacts with the intracellular sequences of GPVI, thereby providing redox signaling pathways and a major source of reactive oxygen species (ROS) generation in platelets.(Arthur et al., 2011) Further, TRAF4 associates with Hic-5 and Pyk2, which constitutively interact with the Src family tyrosine kinase Lyn upon GPVI engagement, leading to propagation of the GPVI signalosome and the recruitment and phosphorylation of Syk.(Arthur et al., 2011; Carrim, Walsh, Consonni, Torti, Berndt & Metharom, 2014) Protein tyrosine phosphorylation has been widely accepted as a central event in the regulation of GPVI signaling in platelets.

Previous studies illustrated that ROS levels are profoundly increased by GPVI stimulation in platelets, whereas intracellular ROS levels do not actively change in response to G protein-coupled receptor (GPCR) agonists such as thrombin and ADP.(Begonja et al., 2005; Krotz et al., 2002; Pignatelli, Pulcinelli, Lenti, Gazzaniga & Violi, 1998) Upon GPVI stimulation, the production of ROS is crucial for propagation of the GPVI signalosome, including PLCγ2 activation, Ca^{2+}mobilization, granule secretion, and αIIbβ3 integrin activation.(Begonja et al., 2005; Krotz et al., 2002; Pignatelli, Pulcinelli, Lenti, Gazzaniga & Violi, 1998) Further, GPVI-mediated ROS production triggers the oxidative inactivation of cytosolic protein tyrosine phosphatases (PTPs) including SH2 domain-containing tyrosine phosphatases 1 and 2 (SHP1 and SHP2), thereby triggering tyrosine phosphorylation-dependent signal transduction.(Jang et al., 2014; Senis, 2013) Therefore, an oxidative submembranous environment in activated platelets would potentially be controlled by the coordinated action of protein tyrosine kinases and PTPs.(Jackson, Schoenwaelder, Yuan, Salem & Cooray, 1996; Tonks & Neel, 1996) However, the underlying mechanism by which ROS regulate the GPVI signaling cascade remains elusive.

A large epidemiological study conducted in the USA regarding the dietary prevention of cardiovascular diseases indicated that thrombogenesis could be reduced by the quality and quantity of dietary fruits and vegetables.(Pallazola et al., 2019; Yamamoto, Ijiri, Ikarugi, Otsui, Inoue & Sakariassen, 2018) Thus, investigating novel natural products and defining novel targets for the prevention of thrombogenesis became important areas of cardiovascular disease research. Flavonoids are ubiquitous secondary metabolites produced under various environmental conditions in fruits and vegetables.(Hertog, Feskens, Hollman, Katan & Kromhout, 1993) Flavonoids are primarily typified by the ability to inhibit enzymes, and they exhibit a number of biological activities such as anti-oxidant and anti-inflammatory properties.(Panche, Diwan & Chandra, 2016; Serafini, Peluso & Raguzzini, 2010) These activities could explain the beneficial effects of flavonoid intake on a variety of human pathologies, including hypertension, inflammatory conditions, and cardiovascular disease.(Comalada et al., 2005) Among the flavonoids, quercetin is the most common flavonoid in nature, and it exists primarily in glycosylated forms such as quercitrin (3-rhamnoside).(Hertog, Feskens, Hollman, Katan & Kromhout, 1993) Quercitrin is known to exhibit biological effects such as anti-oxidant, anti-inflammatory, and anti-apoptotic activities.(Ma, Luo, Jiang & Liu, 2016; Zhi et al., 2016) The sugar moiety of quercitrin usually increases its solubility in polar solvents, consequently resulting in improved absorption.(Gee, DuPont, Rhodes & Johnson, 1998) Thus, quercitrin might be a more potent anti-oxidant than quercetin because of its high bioavailability in the digestive tract.(Dai, Ding, Zhang, Cai & Li, 2013;

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Jo et al., 2008) However, little is known about the biological properties of the glycoside form (quercitrin) compared with the aglycone form (quercetin) because of the lack of commercial standards. Although a variety of pharmacological activities of quercitrin have been studied,(Ma, Luo, Jiang & Liu, 2016; Zhi et al., 2016) the mechanism underlying its potential inhibitory effects on platelet thrombus formation has not been studied previously. Given that GPVI-mediated platelet activation is inhibited by anti-oxidant enzymes, it is suggested that flavonoids are key negative regulators of GPVI-mediated signaling pathways via regulating ROS scavenging. However, the role of flavonoids in GPVI-stimulated platelet activation is unclear. Therefore, the present study aimed to clarify the anti-platelet activity of quercitrin.

In the present study, we demonstrated that quercitrin inhibits thrombus formation *in vivo* and *in vitro*. Quercitrin specifically inhibits platelet activation and aggregation induced by collagen-related peptide (CRP) and U46619. Using biochemical approaches, we revealed that quercitrin acts as a negative regulator of the GPVI-mediated signalosome, probably dependently of GPVI-mediated ROS generation. Further, the anti-oxidant effect of quercitrin is probably derived from the decreased phosphorylation of components of the TRAF4/p47^{phox}/Hic5 signalosome. Studies using a mouse model of ischemia/reperfusion-induced stroke indicated that quercitrin plays a crucial role in stroke-induced brain damage. The tail bleeding time was not significantly higher in quercitrin-treated mice than in control mice. These results demonstrate that quercitrin can potentially exert anti-platelet and antithrombotic effects without affecting hemostasis. Hence, quercitrin could be an effective therapeutic agent for the treatment of thrombotic diseases.

METHODS

Extended methods are given in the Supplement Method.

Statistical analysis. Data analysis was performed using GraphPad Prism 5. Statistical significance was assessed by ANOVA and Tukey's test or Dunnett's test for comparisons of multiple groups or Student's *t*-test for comparisons of two groups. A P value less than 0.05 was considered significant.

RESULTS

Platelet aggregation and ATP secretion are inhibited by treatment with quercitrin

To investigate the effects of quercitrin on platelet function, we first examined platelet aggregation. Stimulation with various agonists including CRP (0.1 μg/ml), U46619 (3 μM), collagen (1 μg/ml), thrombin (0.025 U/ml), and ADP (2.5 μM) led to significant increases in platelet aggregation (Figure 1). Compared with the findings for the vehicle control, quercitrin blocked platelet aggregation induced by intermediate concentrations of CRP ([?] 0.1 μg/ml), U46619 ([?] 3 μM), and collagen ([?] 1 μg/ml) (Figure 1A-1C), whereas such findings were not found for thrombin ([?] 0.025 U/ml) and ADP stimulation ([?] 2.5 μM) (Figure 1D and 1E). Although platelet aggregation was inhibited in the presence of quercitrin upon CRP, U46619, and collagen stimulation, it is possible that the residual activity can overcome when high concentration of agonists ([?] 0.5 μg/ml CRP, [?] 2.5 μg/ml Collagen, and [?] 10 μM U46619, Figure S1). Similar findings were observed in mouse platelets (Figure S2). These results imply that quercitrin exhibits an important effect on the activation of GPVI signaling, although it does not completely block this pathway. A previous study revealed that flavonoids could bind to plasma proteins.(Wright, Spencer, Lovegrove & Gibbins, 2013) Therefore, the effect of quercitrin on platelet activation using PRP was also investigated in comparison with that in washed platelets. We observed that collagen ([?] 1 μg/ml)-stimulated platelet aggregation in PRP was diminished by quercitrin in a concentration-dependent manner similar to the effects of washing platelets (Figure 1F). Consistent with the previous report, CRP stimulation did not induce platelet aggregation in PRP compared with the findings in washed platelets (data not shown). These results suggest that plasma proteins do not influence the effects of quercitrin on platelet aggregation. To further confirm our findings, we examined ADP secretion. In these experiments, CRP, U46619, and collagen also induced [?] 3-fold increases in ADP secretion compared with the control findings (Figure 1A(ii)–1D(ii)). Compared with the results for vehicle-treated platelets, quercitrin-treated platelets exhibited significant defects in ATP secretion induced by CRP (0.1 μg/ml), U46619 (3 μM), and collagen (1 μg/ml) but not by thrombin (Figure 1A(ii)–1D(ii)). These results indicate that quercitrin could regulate platelet aggregation and ATP secretion

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through a signaling pathway induced by both CRP and U46619, but its effects are distinct from those of thrombin.

Χυερςιτριν εξηιβιτς ιμπορταντ εφφεςτ ον Π-σελεςτιν εξποσυρε ανδ αΙΙββ3 ιντεγριν αςτιατιον φολλοωινγ αγονιστ στιμυλατιον

Next, we investigated the effects of quercitrin on P-selectin exposure and αIIbβ3 integrin activation, which are key processes driving the positive feedback cycle of platelet activation. Because quercitrin prevented platelet aggregation and ADP secretion induced by CRP and U46619 stimulation, we examined P-selectin exposure and αIIbβ3 integrin activation using CRP (0.1 μg/ml) or U46619 (3 μM). Following 10 minutes of pre-incubation, quercitrin significantly inhibited CRP- or U46619-induced P-selectin exposure (Figure 2A and 2C) and αIIbβ3 integrin activation (Figure 2B and 2D) in a concentration-dependent manner (10, 20, and 30 μM), whereas quercitrin failed to block the effects of thrombin stimulation (Figure S3). These results suggest that quercitrin selectively inhibits platelet activation through effects on granule secretion and αIIbβ3 integrin activation.

Quercitrin potently inhibits Ca^{2+} mobilization

The underlying mechanism of Ca^{2+} mobilization in the cytosol is crucial for platelet activation involving granule secretion and αIIbβ3 integrin activation.(Stefanini, Roden & Bergmeier, 2009) We examined whether quercitrin affects Ca^{2+} mobilization during platelet activation. We found that compared with the findings in vehicle-treated platelets, quercitrin significantly inhibited intracellular Ca^{2+} release induced by CRP and U46619 stimulation in a concentration-dependent manner (10, 20, and 30 μM) (Figure 3A and 3B). In addition, Ca^{2+} influx induced by CRP was significantly reduced in quercitrin-treated platelets. However, quercitrin did not affect store-operated Ca^{2+} entry (SOCE) induced by U46619 stimulation (Figure 3B). These results suggest that quercitrin inhibits intracellular Ca^{2+} release and SOCE induced by CRP stimulation, but its effects are distinct from those of U46619 stimulation.

The selective effects of quercitrin on GPVI-induced ROS generation in human platelets

Quercitrin is already known to demonstrate free radical scavenging activity;(Li, Jiang, Wang, Liu & Chen, 2016) however, the precise effects of quercitrin on platelet activation are unclear. Therefore, we studied the anti-oxidant effects of quercitrin during the recruitment of platelets induced by CRP stimulation. To determine whether platelet agonists induce ROS (H_2O_2) generation, we first measured intracellular ROS levels via flow cytometry using fluorescent DCFH-DA as a probe. Because quercitrin-treated platelets were selectively defective for CRP- and U46619-induced platelet aggregation and ATP secretion (Figure 1), human platelets were stimulated with CRP (0.1 μg/ml), U46619 (3 μM), or thrombin (0.025 U/ml). Consistent with the aforementioned results, quercitrin treatment selectively inhibited CRP- or U46619-induced intracellular ROS generation (Figure 4A and 4B) relative to the findings in the vehicle controls, whereas the compound exhibited no effects on thrombin-induced ROS generation (Figure S4). These results suggest that the inhibition of intracellular ROS generation is selectively important for the effects of quercitrin on platelet activation. To further examine whether quercitrin alters H_2O_2 accumulation in the extracellular space, we measured extracellular ROS levels using the Amplex Red assay.(Wojtala, Bonora, Malinska, Pinton, Duszynski & Wieckowski, 2014) Compared with the findings for the vehicle control, quercitrin treatment decreased extracellular H_2O_2 levels in response to CRP stimulation in a concentration-dependent manner (Figure 4C). These results suggest that quercitrin regulates ROS generation, which could be important for its inhibitory effects on platelet activation.

Effects of quercitrin on platelet spreading

Upon ligand binding to αIIbβ3 integrin, it mediates outside-in signaling events involved in platelet spreading, thrombus contraction, and clot retraction.(Lee, Fong, King, Brass & Hammer, 2012) Therefore, we examined whether outside-in signaling events are affected by quercitrin using the platelet spreading assay (Figure 5). We found that vehicle- and quercitrin-pretreated platelets adhered to and spread on immobilized fibrinogen (FG) during the 2-hour incubation (Figure 5A). However, the number of adherent platelets was signifi-

cantly inhibited by quercitrin treatment in a concentration-dependent manner (Figure 5B). The spreading of platelets was also reduced by approximately 1.5-fold in quercitrin–pretreated platelets compared with the findings for vehicle platelets (Figure 5C). No differences in lamellipodial actin assembly and surface coverage were observed between vehicle- and quercitrin-treated platelets spread on FG-coated surfaces (Figure 5A and 5D). These results suggest that the effects of quercitrin are likely limited to initial platelet adhesion to the FG-coated surface, and these effects occur through the regulation of the ligand-binding activity of αIIbβ3 integrin.

Quercitrin induces the downregulation of GPVI signaling events and modulates the contribution of TxA$_2$ signaling in human platelets

Since quercitrin significantly reduced platelet aggregation and ATP secretion following CRP stimulation, we investigated the mechanism by which quercitrin regulates CRP-induced GPVI signaling, which activates numerous kinases including Syk, PLCγ2, PI3K, and AKT. To match the experimental condition with platelet aggregation assays, human platelets were stimulated with CRP (0.1 μg/ml) under stirring conditions in an aggregometer and lysed immediately after 5-minute progress. We found that Syk, PLCγ2, PI3K, and AKT were phosphorylated after CRP stimulation in vehicle–pretreated platelets, whereas quercitrin significantly reduced the phosphorylation of Syk, PLCγ2, PI3K, and AKT following CRP stimulation in a concentration-dependent manner (Figure 6A and 6B). Since quercitrin impaired TxA$_2$-induced platelet aggregation and TxA$_2$ reflects the response to collagen-mediated human platelet aggregation,(Cho et al., 2003) we also studied whether quercitrin modulates platelet activation following TxA$_2$ stimulation. The phosphorylation levels of PLCβ3, PI3K, and AKT were elevated in U46619-activated control platelets with stirring, but the phosphorylation patterns were markedly diminished by quercitrin in a concentration-dependent manner (Figure 6C and 6D). These results suggest that quercitrin regulates both GPVI-and TxA2-mediated platelet signaling, thereby regulating platelet activation.

Quercitrin regulates GPVI-mediated platelet activation via PTPs activation

Previous research demonstrated that intracellular ROS participate in the regulation of protein tyrosine phosphorylation by oxidizing the catalytic cysteine residues of PTPs (SHP1, SHP2, and PTEN), which play a negative regulatory role in GPVI-mediated platelet activation.(Jang et al., 2014; Senis, 2013) Since quercitrin serves as an antioxidant, we next examined whether quercitrin affects the activation of PTPs during platelet activation. We found that the phosphorylation levels of SHP1, SHP2, and PTEN were increased in CRP-activated vehicle–pretreated platelets, whereas their phosphorylation was abrogated by quercitrin treatment in a concentration-dependent manner (Figure 6E and 6F). These results suggest that ROS generation upon GPVI stimulation leads to PTP inactivation and promotes tyrosine phosphorylation-based signal transduction, whereas quercitrin, as an anti-oxidant, scavenges ROS and promotes PTP dephosphorylation, thereby reducing GPVI-mediated platelet activation.

Quercitrin alters focal adhesion tyrosine kinase phosphorylation during GPVI-dependent platelet activation

Since the presence of TRAF4/p47^{phox}/Hic5 complex associated with the platelet collagen receptor facilitates GPVI-dependent ROS formation,(Carrim, Walsh, Consonni, Torti, Berndt & Metharom, 2014) we determined whether quercitrin could affect the interaction of GPVI and TRAF4 during platelet activation. We found that the binding of TRAF4 to GPVI was not changed in CRP-activated platelets in the presence or absence of quercitrin, whereas TRAF4 phosphorylation was abrogated in quercitrin-treated platelets (Figure 6G and 6H). Further, because the association of TRAF4 with p47^{phox}(NADPH oxidase organizer subunit) and Hic5 (which is constitutively bound to Syk and Lyn upon GPVI engagement) is involved in GPVI-dependent ROS generation,(Arthur et al., 2011) we also examined the phosphorylation levels of p47^{phox} and Hic5. We found that the phosphorylation levels of p47^{phox} and Hic5 were significantly reduced by quercitrin following CRP stimulation (Figure 6I and 6J). These results suggest that quercitrin does not affect the binding of TRAF4 to GPVI, but it impairs the phosphorylation of members of the TRAF4/p47^{phox}/Hic5 complex associated with the platelet collagen receptor, thereby regulating ROS generation and GPVI-mediated sig-

naling.

Quercitrin inhibits thrombus formation on collagen-coated surfaces under arteriolar shear

To assess the effects of quercitrin on *in vitro* thrombus formation, a flow chamber assay was performed under arteriolar shear (1,000 s^{-1}). In this experiment, the amount of quercitrin administrated was based on a previous finding.(Ma, Luo, Jiang & Liu, 2016) Blood drawn from vehicle-treated mice exhibited widespread coverage with densely packed platelet thrombi on collagen surfaces (Figure 7A). Conversely, blood drawn from quercitrin-treated mice displayed markedly reduced surface coverage (Figure 7B). Moreover, as determined by confocal microscopy, the volume of quercitrin-treated platelet thrombi was significantly lower than that of vehicle-treated platelet thrombi (Figure 7C). These results indicate that the dietary administration of quercitrin inhibits platelet adhesion and aggregation on collagen-coated surfaces under arteriolar shear.

Quercitrin inhibits *in vivo* FeCl$_3$-induced thrombus formation but not hemostasis.

Due to the potent inhibitory effect of quercitrin on platelet function, we further hypothesized that quercitrin possesses anti-thrombotic properties. The potential effect of quercitrin on platelet thrombus formation *in vivo* was investigated via FeCl$_3$-induced carotid artery thrombi, and the time to arterial occlusion was monitored. Carotid occlusion in the control mice occurred at a mean of 7.68 ± 2.19 minutes, whereas in the administration of quercitrin extended the carotid occlusion time to 12.13 ± 3.49 (50 mg/kg, BW) or 14.65 ± 2.51 (100 mg/kg, BW) minutes (Figure 7D). We further examined whether the dietary administration of quercitrin influences hemostatic function. Tail bleeding times were measured by the cessation of bleeding after tail amputation. We observed no statistical differences in tail bleeding time between the quercitrin and control groups (Figure 7E). These results suggest that quercitrin inhibits arterial thrombosis but not hemostasis *in vivo* .

Quercitrin contributes to the pathogenesis of cerebral ischemia/reperfusion injury

Since quercitrin impaired GPVI-mediated platelet activation and genetic and pharmacologic approaches revealed the critical role of GPVI in the pathogenesis of cerebral ischemia/reperfusion injury,(Stegner, Klaus & Nieswandt, 2019) we examined the effect of quercitrin using a murine model of brain injury induced by middle cerebral artery occlusion and reperfusion. Middle cerebral artery occlusion led to neurological deficits as assessed using the Neurological deficit score test, as well as infarction. However, quercitrin (50 or 100 mg/kg, BW) dose-dependently reduced the infarct volume (Figure 8A and 8B) and ameliorated neurological behavior disorder (Figure 8C) compared with the finding in control mice. These results support the protective effects of quercitrin against stroke-induced brain damage.

DISCUSSION

A better understanding of the relationship between flavonoids and platelet function could lead to improved strategies for treating cardiovascular diseases. In this study, we characterized quercitrin, a dietary flavonoid with various pharmacological activities that is widely distributed in nature.(Ma, Luo, Jiang & Liu, 2016; Muzitano et al., 2006; Sanchez de Medina, Vera, Galvez & Zarzuelo, 2002; Zhi et al., 2016) Although quercitrin exhibits weaker effects on the inflammatory response and platelet activation *in vitro* than quercetin, the beneficial effects of quercitrin have not been reproduced *in vivo* .(Comalada et al., 2005) Indeed, quercetin is not well absorbed through the intestines, and thus, its plasma levels are not sufficient for inhibiting platelet function.(Crespy, Morand, Manach, Besson, Demigne & Remesy, 1999; Hubbard, Wolffram, Lovegrove & Gibbins, 2004) Further, previous studies did not explore the mechanism by which quercitrin inhibits platelet activation. In this study, we demonstrated that quercitrin can inhibit platelet aggregation and prevent platelet thrombus formation by suppressing GPVI-mediated signaling and ROS generation. However, platelet activation induced by the GPCR agonist thrombin was not inhibited by quercitrin, indicating that quercitrin inhibits platelet function via GPVI-dependent signaling pathways.

Although a variety of agonists can induce strong platelet aggregation, collagen only substantially stimulated ROS generation in platelets, in accordance with other observations.(Caccese et al., 2000; Krotz et al., 2002)

The binding of collagen to GPVI induced the activation and phosphorylation of PLCγ via numerous tyrosine phosphorylation steps. The inhibition of tyrosine kinases prevented collagen-dependent ROS generation. However, this finding cannot fully explain the observation that only collagen induced an increase of ROS generation, as thrombin is also known to induce the phosphorylation of tyrosine kinases.(Coughlin, 2001; Zielinski, Wachowicz, Saluk-Juszczak & Kaca, 2001) Recent findings demonstrated that TRAF4 directly binds to the cytoplasmic tail of GPVI, thereby providing a mechanism of NADPH oxidase activation(Arthur et al., 2011). Further, p47^{phox}, the cellular subunit of NADPH, binds to TRAF4, which constitutive interacts with Syk and Lyn upon GPVI engagement, thereby activating redox signaling pathways.(Arthur et al., 2011) In our experiments, quercitrin substantially abolished the phosphorylation of members of the TRAF4/p47^{phox}/Hic5 complex without affecting the interaction between TRAF and GPVI (Figure 6G-6J), suggesting that quercitrin may be a negative regulator of activation of the TRAF4/p47^{phox}/Hic5 complex during GPVI-mediated platelet activation. Thus, this finding could explain the differences in ROS generation between the distinct platelet activation pathways mediated by GPVI and PARs. However, we could not rule out the possibility that a low concentration of thrombin can induce phosphorylation of kinases but may not be reached the increase of platelet ROS generation.

Additionally, we observed that quercitrin markedly diminished platelet aggregation and ROS generation not only evoked by CRP stimulation but also by U46619 stimulation in a concentration-dependent manner (Figure 1 and 4). These results are consistent with the effects of the TxA$_2$-induced thromboxane receptor (TP)-ROS signaling pathway,(Muzaffar, Shukla, Lobo, Angelini & Jeremy, 2004) and the binding of TxA$_2$ to TP on platelets induces PLC activation and stimulates inositol 1,4,5-triphosphate and diacylglycerol production, resulting in increased intracellular Ca^{2+} accumulation and ROS generation.(Moers et al., 2003; Offermanns, 2006) Therefore, it appears that quercitrin inhibits TxA$_2$-mediated NADPH oxidase activation and that TxA$_2$-mediated platelet aggregation is also influenced by ROS generation, which is crucial for positive feedback related to platelet activation. However, the relevance of TP-mediated ROS signaling to the pathophysiological actions of TxA$_2$ requires further investigation.

A number of flavonoids have been demonstrated to inhibit various active pathways, likely through the inhibition of tyrosine and lipid kinases involved in platelet responses.(Benavente-Garcia & Castillo, 2008; Wright, Spencer, Lovegrove & Gibbins, 2013) Since quercitrin inhibited CRP-induced platelet aggregation and activation, we also investigated the activation of GPVI signaling proteins. CRP-mediated GPVI signaling induces tyrosine phosphorylation of the Fc receptor γ-chain by Fyn and Lyn, which allows the assembly of Syk and subsequent formation of a complex among LAT, SLP-76, PI3K, PLCγ2, and Btk, leading to activation of the signaling molecules.(Nieswandt & Watson, 2003; Varga-Szabo, Braun & Nieswandt, 2009) In particular, PIP$_3$ produced by PI3K is necessary for the membrane translocation and full activation of PLCγ2 during GPVI-mediated signaling.(Bobe et al., 2001; Watanabe et al., 2003) We found that the phosphorylation of GPVI-activated signaling proteins such as Syk, PLCγ2, PI3K, and AKT was dramatically diminished in a concentration-dependent manner by quercitrin (Figure 6A and 6B). However, their phosphorylation was not changed by other agonists such as thrombin and ADP (data not shown). These data suggest that quercitrin can influence the immediate effectors within the GPVI signaling pathway, which do not participate in activation mechanisms stimulated by other agonists.

Protein tyrosine phosphorylation has been widely accepted to exhibit a central role in the GPVI signaling pathway, and tyrosine phosphorylation has been considered to be controlled by the reconciled action of protein tyrosine kinases and PTPs including SHP1, SHP2, and PTEN.(Senis, 2013) Thus, PTPs are considered important regulators of GPVI-mediated signal transduction. Recent studies indicated that ROS are important for the conformational change-mediated inactivation of PTPs, thereby leading to the phosphorylation of immunoreceptor tyrosine-based inhibition motif-containing receptors.(Senis, 2013) However, we found that the phosphorylation levels of PTEN, SHP1, and SHP2 were reduced by quercitrin, and these changes were correlated with GPVI-mediated signal transduction (Figure 6E and 6F). Thus, consistent with a previous report demonstrating the importance of ROS to PTP activation during platelet activation,(Jang et al., 2014) these findings provide evidence that quercitrin regulates ROS-mediated PTP signaling pathways. We speculate that quercitrin modulates an early stage of GPVI-mediated signal transduction, whereas ROS

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participate in a later stage of the feedback cycle of platelet activation by inactivating PTP. Although the detailed mechanisms remain to be elucidated, our results suggest that ROS generation and kinase activity are mutually regulated.

Overall, our studies provide mechanistic insight into the novel effects of quercitrin on platelet function and ROS generation upon GPVI-mediated platelet activation. Thus, our results provide evidence that quercitrin may be a novel therapeutic agent for the treatment of thrombotic diseases.

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DISCLOSURES

The authors have no conflicting financial interests.

FIGURE LEGENDS

Figure 1. Inhibitory effect of quercitrin on platelet aggregation and ATP secretion following stimulation with numerous agonists. Washed platelets were preincubated with various concentrations of quercitrin (10, 20, and 30 μM) for 5 minutes at 37°C and then stimulated with 0.1 μg/ml CRP (A), 3 μM U46619 (B), 1 μg/ml Collagen (C), 0.025 U/ml thrombin (D), and 2.5 μM ADP (E). Similarly, the effect of quercitrin on PRP was analyzed following stimulation with 1 μg/ml Collagen (F). Aggregation was measured for 5 minutes at 37°C under constant stirring (1,000 rpm) conditions in a platelet aggregometer (Chrono-Log). In the ADP-induced aggregation assay, 30 μg/ml of human FG was added to the platelet suspension before ADP stimulation. The representative aggregation traces were obtained from three independent experiments. (i) Platelet aggregation and quantitative graphs. Data represent the mean ± SD (n = 3). In the ADP secretion, washed platelets were preincubated with various concentrations of quercitrin (10, 20, and 30 μM) for 5 minutes at 37°C before adding a luciferin/luciferase reagent. After the luciferin/luciferase reagent added, platelets were stimulated with 0.1 μg/ml CRP (A), 3μM U46619 (B), 1 μg/ml Collagen (C), and 0.025 U/ml thrombin (D). (ii) ATP secretion was measured using a luminometer. Data represent the mean ± SD (n = 3). *:P<0.05, **:P<0.01, and ***:P<0.001 versus vehicle control after ANOVA and Dunnett's test.

Figure 2. Quercitrin modulates αΙΙββ3 ιντεγριν αςτιατιον ανδ Π-σελεςτιν εξποσυρε δυρινγ πλατελετ αςτιατιον. The inhibitory effect of quercitrin on CRP or U46619 induced αIIbβ3 integrin activation (A and C) and P-selectin exposure (B and D) was measured by flow cytometry. Washed human platelets were pre-treated with various concentration of quercitrin (10, 20, and 30 μM) for 5 minutes at 37°C and stimulated with 0.1 μg/ml CRP (A and B) or 3 μM U46619 (C and D). Binding of anti-activated αIIbβ3 (JON/A) and anti-P-selectin antibodies to platelets was calculated by the ratio of the geometric mean fluorescence intensity (MFI) value of antibodies to that of control IgG. Data represent mean ± SD (n = 3). *:P<0.05, **:P<0.01, and ***:P<0.001 versus vehicle control after ANOVA and Dunnett's test.

Figure 3. Quercitrin regulates Ca^{2+} mobilization during platelet activation. Human platelets were resuspended in HEPES-Tyrode buffer without 1 mM $CaCl_2$ and preincubated with various concentrations of quercitrin (10, 20, and 30 μM) for 5 minutes at 37°C and incubated with a calcium-sensitive dye for 30 minutes at 37°C in the dark. After treatment with a Ca^{2+} dye, platelets were stimulated with 0.1 μg/ml CRP (A) or 3 μM U46619 (B) for 10 minutes and 2 mM $CaCl_2$ was then added. Intracellular Ca^{2+} release (i) and influx (ii) were measured and quantified by the AUC (arbitrary units). Quantitative data represent the mean ± SD (n = 3). *:P<0.05 and **:P<0.01 versus vehicle control after ANOVA and Dunnett's test.

Figure 4. Quercitrin modulates intracellular and extracellular H_2O_2 following agonist stimulation. Vehicle control (0.02% DMSO) and quercitrin (10, 20, and 30 μM) were incubated with H_2DCFDA and stimulated with 0.1 μg/ml CRP (A) or 3 μM U46619 (B). DCF fluorescence was measured using flow cytometry and is quantified as mean ± SD (n = 3). *:P<0.05 versus vehicle control after ANOVA and Dunnett's test. Extracellular H_2O_2 levels were measured in CRP-stimulated platelets using the Amplex Red assay (C). The fluorescence signal was measured by a microplate reader and is quantified as mean ± SD (n = 3). **:P<0.01 and ***:P<0.001 versus vehicle control after ANOVA and Dunnett's test. ###:P<0.001 between resting and stimulated control group.

Figure 5. The inhibitory effect of quercitrin on platelet spreading on immobilized fibrinogen. Human platelets, 8 x 10^6 platelets in 0.4 ml, were pre-treated with various concentrations of quercitrin (10, 20, and 30 μM) or vehicle control (0.02% DMSO) and incubated on FG-coated surfaces for 2 hours at 37°C. Adherent and spread platelets were stained with rhodamine-conjugated phalloidin. (A) Representative images. Bar = 10 μm. (B) Number of adherents (but not spread, gray bars) and fully spread (white bars). Platelet spreading was analyzed by the surface area (C) which was measured by the number of pixels divided by the number of platelets (D) in the field. *:P<0.05, **:P<0.01, and ***:P<0.001 versus vehicle control after ANOVA and Dunnett's test. Data represent mean ± SD (n = 5).

Figure 6. Quercitrin plays a crucial role in the GPVI-mediated signalosome. Human platelets were pre-treated with various concentrations of quercitrin (10, 20, and 30 μM), and stimulated with 0.1 μg/ml CRP (A, E, and I) or 3 μM U46619 (C) under stirring conditions (1,000 rpm) in an aggregometer. An equal amount of cell lysate protein (30 μg) was immunoblotted, followed by densitometry (arbitrary unit (AU)). Representative blots (A, C, E, and I). Quantitative graphs (B, D, F, and J). (G-H) Human platelets were stimulated by 0.1 μg/ml CRP for 5 minutes under stirring conditions. The lysates were immunoprecipitated with control IgG or antibodies against GPVI or 4G10, followed by immunoblotting and densitometry (n = 3). Data represent the mean ± SD (n = 3-4). ***:P<0.01 versus vehicle control (unstimulated) after Student's t-test, and #:P<0.05, ##:P<0.01 and ###:P<0.001 versus vehicle control (stimulated) after ANOVA and Turkey's test.

Figure 7. Role of Quercitrin in thrombus formation and tail bleeding time. Blood drawn from vehicle or quercitrin (50 or 100 mg/kg, BW) treated mice were perfused over collagen-coated surfaces at a wall shear rate of 1000 s^{-1} for 1 minute through the chamber. Adherent thrombi were stained with rhodamine-conjugated phalloidin and analyzed as described in Method. (A) Representative images. Bar = 10 μm. (B) Surface coverage and (C) thrombus volume were measured and presented as mean ± SD (n = 3). (D) $FeCl_3$-induced arterial thrombus formation was performed as described in Methods. Mouse carotid artery was isolated and treated with 10% $FeCl_3$ for 2 minutes, and blood flow traces were monitored until stable occlusion took place using a blood flowmeter. (E) Tails of vehicle or quercitrin treated mice were amputated, and bleeding time was monitored as described in Methods. Horizontal bars represent the median of occlusion and bleeding times for each group of animals (n = 10). *:P<0.05 and ***:P<0.001 versus vehicle control after ANOVA and Dunnett's test.

Figure 8. Quercitrin is important for cerebral ischemia/reperfusion injury *in vivo*.

Cerebral ischemia/reperfusion injury was induced as described in Methods. (A) Representative pictures of brain sections stained with 2,3,5-triphenyl tetrazolium chloride. (B) The infarct volume was measured by Image J. (C) The Neurological deficit score test was used to analyze neurological behavior disorder in the surviving animals 24 hours after MCAO and reperfusion. Neurological findings were scored in a 5-point scale as follows: no neurological deficit=0, failure to extend right forepaw fully=1, circling to the right=2, falling to extend right=3 and no spontaneous walking with a depressed level of consciousness=4. The bars represent the median of the interquartile range for each group of animals (n = 5). *:P<0.05 and ***:P<0.001 versus vehicle control after ANOVA and Turkey's test.

REFERENCES

Arthur JF, Shen Y, Gardiner EE, Coleman L, Murphy D, Kenny D, *et al.* (2011). TNF receptor-associated

factor 4 (TRAF4) is a novel binding partner of glycoprotein Ib and glycoprotein VI in human platelets. J Thromb Haemost 9: 163-172.

Begonja AJ, Gambaryan S, Geiger J, Aktas B, Pozgajova M, Nieswandt B, *et al.* (2005). Platelet NAD(P)H-oxidase-generated ROS production regulates alphaIIbbeta3-integrin activation independent of the NO/cGMP pathway. Blood 106: 2757-2760.

Benavente-Garcia O, & Castillo J (2008). Update on uses and properties of citrus flavonoids: new findings in anticancer, cardiovascular, and anti-inflammatory activity. J Agric Food Chem 56: 6185-6205.

Bobe R, Wilde JI, Maschberger P, Venkateswarlu K, Cullen PJ, Siess W, *et al.* (2001). Phosphatidylinositol 3-kinase-dependent translocation of phospholipase Cgamma2 in mouse megakaryocytes is independent of Bruton tyrosine kinase translocation. Blood 97:678-684.

Caccese D, Pratico D, Ghiselli A, Natoli S, Pignatelli P, Sanguigni V, *et al.* (2000). Superoxide anion and hydroxyl radical release by collagen-induced platelet aggregation–role of arachidonic acid metabolism. Thromb Haemost 83: 485-490.

Carrim N, Walsh TG, Consonni A, Torti M, Berndt MC, & Metharom P (2014). Role of focal adhesion tyrosine kinases in GPVI-dependent platelet activation and reactive oxygen species formation. PLoS One 9: e113679.

Cho MJ, Liu J, Pestina TI, Steward SA, Thomas DW, Coffman TM, *et al.* (2003). The roles of alpha IIb beta 3-mediated outside-in signal transduction, thromboxane A2, and adenosine diphosphate in collagen-induced platelet aggregation. Blood 101: 2646-2651.

Comalada M, Camuesco D, Sierra S, Ballester I, Xaus J, Galvez J, *et al.* (2005). In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-kappaB pathway. Eur J Immunol 35:584-592.

Coughlin SR (2001). Protease-activated receptors in vascular biology. Thromb Haemost 86: 298-307.

Crespy V, Morand C, Manach C, Besson C, Demigne C, & Remesy C (1999). Part of quercetin absorbed in the small intestine is conjugated and further secreted in the intestinal lumen. Am J Physiol 277:G120-126.

Dai X, Ding Y, Zhang Z, Cai X, & Li Y (2013). Quercetin and quercitrin protect against cytokineinduced injuries in RINm5F beta-cells via the mitochondrial pathway and NF-kappaB signaling. Int J Mol Med 31: 265-271.

Furie B, & Furie BC (2008). Mechanisms of thrombus formation. N Engl J Med 359: 938-949.

Gee JM, DuPont MS, Rhodes MJ, & Johnson IT (1998). Quercetin glucosides interact with the intestinal glucose transport pathway. Free Radic Biol Med 25: 19-25.

Hertog MG, Feskens EJ, Hollman PC, Katan MB, & Kromhout D (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 342: 1007-1011.

Hubbard GP, Wolffram S, Lovegrove JA, & Gibbins JM (2004). Ingestion of quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in humans. J Thromb Haemost 2: 2138-2145.

Jackson SP, Schoenwaelder SM, Yuan Y, Salem HH, & Cooray P (1996). Non-receptor protein tyrosine kinases and phosphatases in human platelets. Thromb Haemost 76: 640-650.

Jang JY, Min JH, Chae YH, Baek JY, Wang SB, Park SJ, *et al.*(2014). Reactive oxygen species play a critical role in collagen-induced platelet activation via SHP-2 oxidation. Antioxid Redox Signal 20: 2528-2540.

Jo HY, Kim Y, Nam SY, Lee BJ, Kim YB, Yun YW, *et al.* (2008). The inhibitory effect of quercitrin gallate on iNOS expression induced by lipopolysaccharide in Balb/c mice. J Vet Sci 9: 267-272.

Krotz F, Sohn HY, Gloe T, Zahler S, Riexinger T, Schiele TM, *et al.* (2002). NAD(P)H oxidase-dependent platelet superoxide anion release increases platelet recruitment. Blood 100**:** 917-924.

Lee D, Fong KP, King MR, Brass LF, & Hammer DA (2012). Differential dynamics of platelet contact and spreading. Biophys J 102**:**472-482.

Li X, Jiang Q, Wang T, Liu J, & Chen D (2016). Comparison of the Antioxidant Effects of Quercitrin and Isoquercitrin: Understanding the Role of the 6''-OH Group. Molecules 21.

Ma JQ, Luo RZ, Jiang HX, & Liu CM (2016). Quercitrin offers protection against brain injury in mice by inhibiting oxidative stress and inflammation. Food Funct 7**:** 549-556.

Moers A, Nieswandt B, Massberg S, Wettschureck N, Gruner S, Konrad I, *et al.* (2003). G13 is an essential mediator of platelet activation in hemostasis and thrombosis. Nat Med 9**:** 1418-1422.

Munnix IC, Strehl A, Kuijpers MJ, Auger JM, van der Meijden PE, van Zandvoort MA, *et al.* (2005). The glycoprotein VI-phospholipase Cgamma2 signaling pathway controls thrombus formation induced by collagen and tissue factor in vitro and in vivo. Arterioscler Thromb Vasc Biol 25**:** 2673-2678.

Muzaffar S, Shukla N, Lobo C, Angelini GD, & Jeremy JY (2004). Iloprost inhibits superoxide formation and gp91phox expression induced by the thromboxane A2 analogue U46619, 8-isoprostane F2alpha, prostaglandin F2alpha, cytokines and endotoxin in the pig pulmonary artery. Br J Pharmacol 141**:** 488-496.

Muzitano MF, Cruz EA, de Almeida AP, Da Silva SA, Kaiser CR, Guette C, *et al.* (2006). Quercitrin: an antileishmanial flavonoid glycoside from Kalanchoe pinnata. Planta Med 72**:** 81-83.

Nieswandt B, & Watson SP (2003). Platelet-collagen interaction: is GPVI the central receptor? Blood 102**:** 449-461.

Offermanns S (2006). Activation of platelet function through G protein-coupled receptors. Circ Res 99**:** 1293-1304.

Pallazola VA, Davis DM, Whelton SP, Cardoso R, Latina JM, Michos ED, *et al.* (2019). A Clinician's Guide to Healthy Eating for Cardiovascular Disease Prevention. Mayo Clin Proc Innov Qual Outcomes 3**:** 251-267.

Panche AN, Diwan AD, & Chandra SR (2016). Flavonoids: an overview. J Nutr Sci 5**:** e47.

Pignatelli P, Pulcinelli FM, Lenti L, Gazzaniga PP, & Violi F (1998). Hydrogen peroxide is involved in collagen-induced platelet activation. Blood 91**:** 484-490.

Sanchez de Medina F, Vera B, Galvez J, & Zarzuelo A (2002). Effect of quercitrin on the early stages of hapten induced colonic inflammation in the rat. Life Sci 70**:** 3097-3108.

Senis YA (2013). Protein-tyrosine phosphatases: a new frontier in platelet signal transduction. J Thromb Haemost 11**:** 1800-1813.

Serafini M, Peluso I, & Raguzzini A (2010). Flavonoids as anti-inflammatory agents. Proc Nutr Soc 69**:** 273-278.

Stefanini L, Roden RC, & Bergmeier W (2009). CalDAG-GEFI is at the nexus of calcium-dependent platelet activation. Blood 114**:**2506-2514.

Stegner D, Klaus V, & Nieswandt B (2019). Platelets as Modulators of Cerebral Ischemia/Reperfusion Injury. Front Immunol 10**:** 2505.

Tonks NK, & Neel BG (1996). From form to function: signaling by protein tyrosine phosphatases. Cell 87**:** 365-368.

Varga-Szabo D, Braun A, & Nieswandt B (2009). Calcium signaling in platelets. J Thromb Haemost 7**:** 1057-1066.

Watanabe N, Nakajima H, Suzuki H, Oda A, Matsubara Y, Moroi M, *et al.* (2003). Functional phenotype of phosphoinositide 3-kinase p85alpha-null platelets characterized by an impaired response to GP VI stimulation. Blood 102**:** 541-548.

Wojtala A, Bonora M, Malinska D, Pinton P, Duszynski J, & Wieckowski MR (2014). Methods to monitor ROS production by fluorescence microscopy and fluorometry. Methods Enzymol 542**:** 243-262.

Wright B, Spencer JP, Lovegrove JA, & Gibbins JM (2013). Insights into dietary flavonoids as molecular templates for the design of anti-platelet drugs. Cardiovasc Res 97**:** 13-22.

Yamamoto J, Ijiri Y, Ikarugi H, Otsui K, Inoue N, & Sakariassen KS (2018). Prevention of thrombotic disorders by antithrombotic diet and exercise: evidence by using global thrombosis tests. Future Sci OA 4**:** FSO285.

Yeung J, Li W, & Holinstat M (2018). Platelet Signaling and Disease: Targeted Therapy for Thrombosis and Other Related Diseases. Pharmacol Rev 70**:** 526-548.
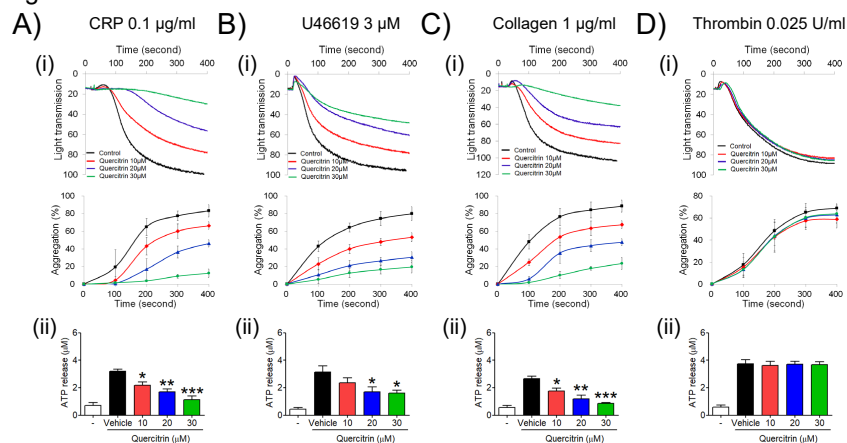
Zhi K, Li M, Bai J, Wu Y, Zhou S, Zhang X, *et al.* (2016). Quercitrin treatment protects endothelial progenitor cells from oxidative damage via inducing autophagy through extracellular signal-regulated kinase. Angiogenesis 19**:** 311-324.

Zielinski T, Wachowicz B, Saluk-Juszczak J, & Kaca W (2001). The generation of superoxide anion in blood platelets in response to different forms of Proteus mirabilis lipopolysaccharide: effects of staurosporin, wortmannin, and indomethacin. Thromb Res 103**:**149-155.

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Figure 1

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Figure S1

A) CRP 0.5 µg/ml   B) U46619 10 µM   B) Collagen 3 µg/ml

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