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Mechanisms Involved in the Antiplatelet Activity of Rutin, a Glycoside of the Flavonol Quercetin, in Human Platelets

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The aim of this study was to systematically examine the inhibitory mechanisms of rutin, a well-known flavonoid in platelet aggregation. In this study, rutin concentration-dependently (250 and 290 μM) inhibited platelet aggregation in human platelets stimulated by agonists (i.e., collagen). Rutin (250 and 290 μM) did not significantly interfere with the binding of FITC−triflavin to the glycoprotein IIb/IIIa complex in human platelets. Rutin (250 and 290 μM) markedly inhibited intracellular Ca^{2+} mobilization and thromboxane A_2 formation in human platelets stimulated by collagen. Rapid phosphorylation of a platelet protein of M_r 47000 (P47), a marker of protein kinase C activation, was triggered by collagen (1 μg/mL). This phosphorylation was markedly inhibited by rutin (250 and 290 μM). On the other hand, rutin (250 and 290 μM) did not significantly increase the formations of cyclic AMP and nitric oxide/cyclic GMP in platelets. In conclusion, these results indicate that the antiplatelet activity of rutin may involve the following pathways: rutin inhibited the activation of phospholipase C, followed by inhibition of protein kinase C activity and thromboxane A_2 formation, thereby leading to inhibition of the phosphorylation of P47 and intracellular Ca^{2+} mobilization, finally resulting in inhibition of platelet aggregation.

KEYWORDS: Rutin; platelet aggregation; phospholipase C; protein kinase C; thromboxane A_2

INTRODUCTION

It is known that polyphenols are a large family of natural compounds derived from various plant sources. Recent studies in vitro and in vivo show that some polyphenols exhibit antioxidant and free radical-scavenging properties (*1*). Therefore, dietary polyphenolics, which possess antioxidant activity, may play a role in human health, particularly in diseases believed to involve, in part, oxidation, such as coronary heart disease, inflammation, and mutagenesis leading to carcinogenesis (*2*, *3*). Rutin, a glycoside of the flavonol quercetin, is one of the major polyphenols. This compound has been extensively studied and is known to exhibit antitumor (*4*), anti-inflammatory (*5*), antiplatelet (*6*), and vasodilation (*7*) activities.

Intravascular thrombosis is one generator of a wide variety of cardiovascular diseases. The initiation of an intraluminal thrombosis is believed to involve platelet adherence and aggregation. In normal circulation, platelets cannot aggregate by themselves. However, when a blood vessel is damaged, platelets adhere to the disrupted surface, and the adherent platelets release some biologically active constituents and aggregate (*8*). Thus, platelet aggregation may play a crucial role in the atherothrombotic process. Indeed, antiplatelet agents (e.g., ticlopidine and aspirin) have been shown to reduce the incidence of stroke in high-risk patients (*9*).

Rutin has been shown to inhibit platelet aggregation in both in vitro and ex vivo experiments (*6*, *7*, *10*). However, no data are available concerning the detailed effects of rutin in platelet aggregation. Therefore, the inhibitory mechanisms of rutin underlying the signaling pathways in platelets remain obscure. We therefore systematically examined the influence of rutin in washed human platelets and utilized the findings to characterize the mechanisms involved in this influence.

MATERIALS AND METHODS

Materials. Collagen (type I, bovine achilles tendon), rutin, sodium citrate, luciferin−luciferase, indomethacin, myoinositol, prostaglandin E_1 (PGE_1), arachidonic acid, apyrase, and heparin were purchased from Sigma Chemical Co. (St. Louis, MO). Fura 2-AM and fluorescein isothiocyanate (FITC) were purchased from Molecular Probes (Eugene, OR). *Trimeresurus flavoviridis* venom was purchased from Latoxan (Rosans, France). Myo-2-[^{3}H]inositol and thromboxane B_2, cyclic AMP, andGMP EIA kits were purchased from Amersham (Buckinghamshire, HP, U.K.). VCl_3 was obtained from Aldrich (Milwaukee, WI). Rutin was dissolved in dimethyl sulfoxide (DMSO) and stored at −4 °C until use.

Preparation of Human Platelet Suspensions. Human platelet suspensions were prepared as previously described (*11*). In this study, human volunteers gave informed consent. In brief, blood was collected from healthy human volunteers who had taken no medicine during the preceding 2 weeks and was mixed with acid/citrate/glucose (9:1, v/v).

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After centrifugation at 120*g* for 10 min, the supernatant (platelet-rich plasma; PRP) was supplemented with PGE_1 (0.5 μM) and heparin (6.4 IU/mL), then incubated for 10 min at 30 °C, and centrifuged at 500*g* for 10 min. The washed platelets were finally suspended in Tyrode's solution containing bovine serum albumin (BSA) (3.5 mg/mL) and adjusted to a concentration of 4.5×10^8 platelets/mL. The final concentration of Ca^{2+} in Tyrode's solution was 1 mM.

Platelet Aggregation. The turbidimetric method was applied to measure platelet aggregation (*11*), using a Lumi-Aggregometer (Payton, Canada). Platelet suspensions (4.5×10^8 platelets/mL, 0.4 mL) were prewarmed to 37 °C for 2 min (stirring at 1200 rpm) in a silicone-treated glass cuvette. Rutin (250 and 290 μM) was added 3 min before the addition of platelet aggregation inducers (i.e., collagen). The reaction was allowed to proceed for at least 6 min, and the extent of aggregation was expressed in light transmission units. While ATP release was measured, 20 μL of a luciferin−luciferase mixture was added 1 min before the addition of agonists, and ATP release was compared with that of the control.

Analysis of the Platelet Surface Glycoprotein IIb/IIIa Complex by Flow Cytometry. Triflavin, a specific fibrinogen receptor (glycoprotein IIb/IIIa complex) antagonist, was prepared as previously described (*12*). Fluorescence-conjugated triflavin was also prepared as previously described (*13*). The final concentration of FITC-conjugated triflavin was adjusted to 1 mg/mL. Human platelet suspensions were prepared as described above. Aliquots of platelet suspensions (4.5×10^8/mL) were preincubated with rutin (250 and 290 μM) for 3 min, followed by the addition of 2 μL of FITC−triflavin. The suspensions were then incubated for another 5 min, and the volume was adjusted to 1 mL/tube with Tyrode's solution. The suspensions were then assayed for fluorescein-labeled platelets with a flow cytometer (Becton Dickinson, FACScan Systems, San Jose, CA). Data were collected from 50000 platelets per experimental group. All experiments were repeated at least four times to ensure reproducibility.

Measurement of Platelet $[Ca^{+2}]i$ Mobilization by Fura 2-AM Fluorescence. Citrated whole blood was centrifuged at 120*g* for 10 min. The supernatant was protected from light and incubated with Fura 2-AM (5 μM) at 37 °C for 1 h. Human platelets were then prepared as described above. Finally, the external Ca^{2+} concentration of the platelet suspensions was adjusted to 1 mM. The rise in $[Ca^{2+}]i$ was measured using a fluorescence spectrophotometer (CAF 110, Jasco) at excitation wavelengths of 340 and 380 nm and at an emission wavelength of 500 nm. $[Ca^{2+}]i$ was calculated from the fluorescence, using 224 nM as the Ca^{2+}−Fura 2 dissociation constant (*14*).

Measurement of Thromboxane B_2 Formation. Washed human platelet suspensions (4.5×10^8/mL) were preincubated for 3 min in the presence or absence of rutin (250 and 290 μM) before the addition of collagen (1 μg/mL). Six minutes after the addition of agonists, 2 mM EDTA and 50 μM indomethacin were added to the reaction suspensions. The vials were then centrifuged for 3 min at 15000*g*. The thromboxane B_2 (TxB_2) levels of the supernatants were measured using an EIA kit according to the instructions of the manufacturer.

Estimation of Platelet Cyclic AMP and Cyclic GMP Formations. The method of Karniguian et al. (*15*) was followed. In brief, platelet suspensions were warmed to 37 °C for 1 min, and then either PGE_1 (10 μM), nitroglycerin (10 μM), or rutin (250 and 290 μM) was added and incubated for 6 min. The incubation was stopped, and the solution was immediately boiled for 5 min. After cooling to 4 °C, the precipitated protein was collected as sediment after centrifugation. Fifty microliters of supernatant was used to determine the cyclic AMP and cyclic GMP contents by EIA kits following acetylation of the samples as described by the manufacturer.

Estimation of Nitrate in Human Platelet Suspensions. NO was assayed in platelet suspensions as previously described (*16*). In brief, platelet suspensions (1×10^9/mL) were preincubated with collagen (1 μg/mL) or rutin (250 and 290 μM) for 3 min, respectively, followed by centrifugation. The amount of nitrate in the platelet suspensions (10 μL) was measured by adding a reducing agent (0.8% VCl_3 in 1 M HCl) to the purge vessel to convert nitrate to NO, which was stripped from the platelet suspensions by a helium purge gas. The NO was then drawn into a Sievers nitric oxide analyzer (Sievers 280 NOA, Sievers Inc., Boulder, CO). Nitrate concentrations were calculated by comparison with standard solutions of sodium nitrate.

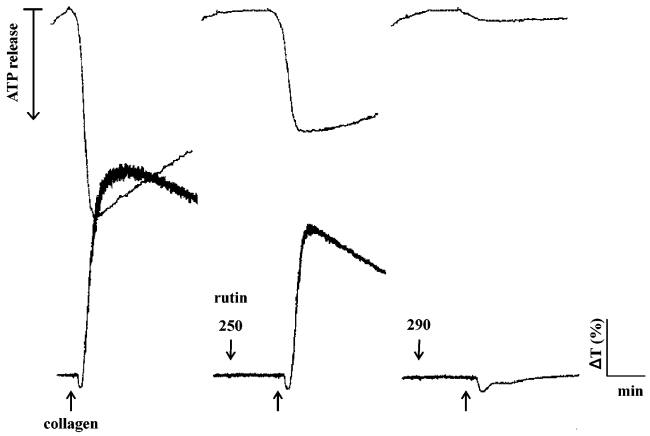

Figure 1. Tracing curves of rutin on collagen (1 μg/mL)-induced aggregation and ATP release in washed human platelets. Platelets were preincubated with rutin (250 and 290 μM) for 3 min, and then collagen was added to trigger aggregation (lower tracings) and ATP release (upper tracings). A luciferin–luciferase mixture (20 μL) was added 1 min before collagen addition to measure the ATP-release reactions. The profiles are representative examples of five similar experiments.

Measurement of Protein Kinase C Activity. Washed human platelets (2×10^9/mL) were incubated for 60 min at 37 °C with phosphorus-32 (0.5 mCi/mL). Platelet suspensions were next washed twice with Tris−saline buffer. The ^{32}P-labeled platelets were preincubated with rutin (250 and 290 μM) in an aggregometer at 37 °C for 3 min, and then collagen (1 μg/mL) was added for 1 min to trigger protein kinase C activation. Activation was terminated by the addition of Laemmli sample buffer, followed by electrophoresis (12.5%, w/v) as described previously (*17*). The gels were dried, and the relative intensities of the radioactive bands were analyzed using a Bio-imaging analyzer system (FAL2000, Fuji, Tokyo, Japan) and expressed as photostimulated luminescence (PSL) per millimeter.

Statistical Analysis. Experimental results are expressed as the means ± SEM and are accompanied by the number of observations. Data were assessed by the method of analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared using the Newman−Keuls method. A p value of <0.05 was considered to be statistically significant.

RESULTS

Effect of Rutin on Platelet Aggregation in Human Platelet Suspensions. Rutin (250 and 290 μM) concentration-dependently inhibited platelet aggregation stimulated by collagen (1 μg/mL) (**Figure 1**), thrombin (0.1 unit/mL), and arachidonic acid (60 μM) in human platelets (data not shown). Furthermore, rutin inhibited the ATP-release reaction when stimulated by agonists (i.e., collagen) (**Figure 1**). On the other hand, its solvent control (0.5% DMSO) did not significantly affect platelet aggregation stimulated by agonists (data not shown). In the following experiments, we used collagen as an agonist to explore the inhibitory mechanisms of rutin in platelet aggregation.

Effect of Rutin on Collagen-Induced Conformational Change of the Glycoprotein IIb/IIIa Complex in Human Platelets. Triflavin is an Arg-Gly-Asp-containing antiplatelet peptide purified from *T. flavoviridis* snake venom (*12*). Triflavin inhibits platelet aggregation through direct interference with fibrinogen binding to the glycoprotein IIb/IIIa complex ($\alpha_{IIb}\beta_3$ integrin) (*12*). There is now a multitude of evidence suggesting that the binding of fibrinogen to the glycoprotein IIb/IIIa complex is the final common pathway for agonist-induced platelet aggregation. Therefore, we decided to further evaluate

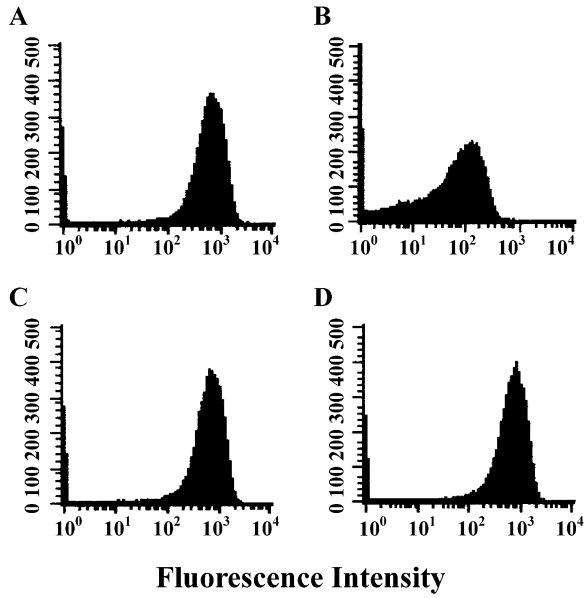

Figure 2. Flow cytometric analysis of FITC–triflavin binding to human platelets in the absence or presence of various concentrations of rutin (250 and 290 μM): (**A**) solid line represents the fluorescence profiles of FITC–triflavin (2 μg/mL) in the absence of rutin as a positive control; (**B**) in the presence of EDTA (5 mM) as the negative control; or in the presence of rutin (**C**) (250 μM) and (**D**) (290 μM). The profiles are representative examples of four similar experiments.

whether rutin binds directly to the platelet glycoprotein IIb/IIIa complex, leading to inhibition of platelet aggregation induced by agonists.

In this study, the relative intensity of fluorescence of FITC−triflavin (2 μg/mL) bound directly to collagen (1 μg/mL)-activated platelets was 581.5 ± 54.2 (**Figure 2A**), and it was markedly reduced in the presence of 5 mM EDTA (negative control, 75.0 ± 1.9, $p < 0.001$) (**Figure 2B**). Rutin (250 and 290 μM) did not significantly inhibit FITC−triflavin binding to the glycoprotein IIb/IIIa complex in platelet suspensions (250 μM, 520.8 ± 45.6; 290 μM, 552.3 ± 64.4) (**Figure 2C**,**D**), indicating that the mechanism of rutin's inhibitory effect on platelet aggregation does not involve directly binding to the glycoprotein IIb/IIIa complex.

Effect of Rutin on [Ca^{2+}]i Mobilization. As shown in **Figure 3**, collagen (1 μg/mL) evoked an increase of [Ca^{2+}]i from 28.1 ± 4.5 to 287.5 ± 27.6 nM. This collagen-evoked increase in [Ca^{2+}]i was markedly inhibited in the presence of rutin (250 μM, 62.7 ± 8.1%; 290 μM, 81.1 ± 6.3%). This suggests that rutin exerts an inhibitory effect on [Ca^{2+}]i mobilization in human platelets stimulated by collagen.

Effect of Rutin on Thromboxane B_2 Formation. As shown in **Table 1**, resting platelets produced relatively little TxB_2 compared with collagen-activated platelets. PGE_1 (10 μM) inhibited TxB_2 formation in collagen-activated platelets by 81% (data not shown). Furthermore, results obtained using various concentrations of rutin indicated that rutin (250 and 290 μM) concentration-dependently inhibited TxB_2 formation in platelets stimulated by collagen (1 μg/mL) about 41 and 46%, respectively (**Table 1**). This result suggests that rutin exerts an inhibitory effect on TxA_2 formation.

Effect of Rutin on Cyclic AMP, Cyclic GMP, and Nitrate Formations in Washed Human Platelets. The level of cyclic AMP in unstimulated platelets was low (2.5 ± 0.8 pmol/mL). Addition of PGE_1 (10 μM) increased the cyclic AMP level to 25.5 ± 1.0 pmol/mL (**Table 2**). When platelet suspensions were

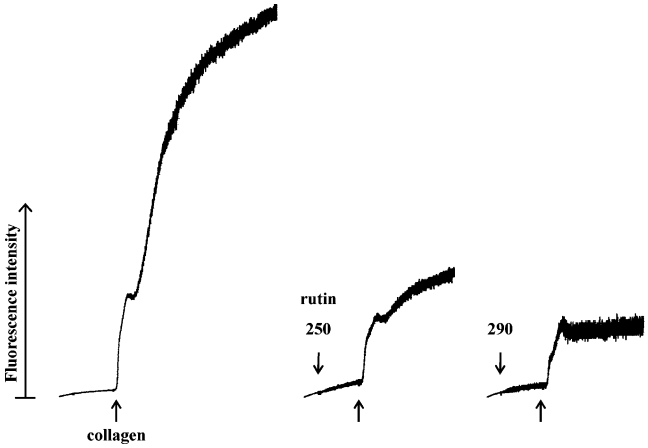

Figure 3. Effect of rutin on collagen-induced intracellular Ca^{2+} mobilization in Fura 2-AM-loaded human platelets. Platelet suspensions were preincubated with Fura 2-AM (5 μM), followed by the addition of collagen (1 μg/mL) in the absence or presence of rutin (250 and 290 μM), which was added 3 min prior to the addition of collagen. The profiles are representative examples of four similar experiments.

Table 1. Effect of Rutin on Thromboxane B_2 Formation Stimulated by Collagen in Washed Human Platelets[a]

	concn	TxB_2[b] (ng/mL)
resting		3.1 ± 0.2
collagen	1 μg/mL	38.7 ± 5.1**
+ rutin	250 μM	22.8 ± 1.6[#]
	290 μM	20.9 ± 2.7[#]

[a] Platelet suspensions were preincubated with rutin (250 and 290 μM) for 3 min at 37 °C, and then collagen (1 μg/mL) was added to trigger thromboxane B_2 formation. Data are presented as the means ± SEM ($n = 5$). [b] **, $p < 0.001$, as compared with the resting group; [#], $p < 0.05$ as compared with the collagen group.

Table 2. Effect of Rutin on Cyclic AMP, Nitrate, and Cyclic GMP Formations in Washed Human Platelets[a]

	concn	cyclic AMP[b] (pmol/mL)	nitrate[b] (μM)	cyclic GMP (pmol/mL)
resting		2.5 ± 0.8	4.4 ± 0.3	0.5 ± 0.1
prostaglandin E_1	10 μM	25.5 ± 1.0**		
collagen	1 μg/mL		7.8 ± 0.3**	
nitroglycerin	10 μM		1.2 ± 0.2*	
rutin	250 μM	2.1 ± 1.0	3.9 ± 0.3	0.5 ± 0.1
	290 μM	1.4 ± 0.4	4.3 ± 0.4	0.5 ± 0.0

[a] Platelet suspensions were preincubated with rutin (250 and 290 μM) for 3 min at 37 °C. Addition of prostaglandin E_1, collagen, and nitroglycerin into the platelet suspensions served as positive control of cyclic AMP, nitrate, and cyclic GMP formations, respectively. Data are presented as the means ± SEM ($n = 4$). [b] *, $p < 0.05$, and **, $p < 0.001$, as compared with the resting groups.

preincubated with various concentrations of rutin (250 and 290 μM) for 3 min at 37 °C, we found that rutin did not significantly increase the cyclic AMP level (**Table 2**). We also performed similar studies measuring cyclic GMP response. The level of cyclic GMP in unstimulated platelets was very low, but when nitroglycerin (10 μM) was added to the platelet suspensions, the cyclic GMP level increased from the resting level to 1.2 ± 0.3 pmol/mL (**Table 2**). However, addition of rutin (250 and 290 μM) resulted in no significant increase in platelet cyclic GMP levels (0.5 ± 0.1 and 0.5 ± 0.0 pmol/mL) (**Table 2**).

On the other hand, NO was quantified using a sensitive and specific ozone redox-chemiluminescence detector. As shown in **Table 2**, collagen (1 μg/mL) caused a ~1.8-fold rise in nitrate

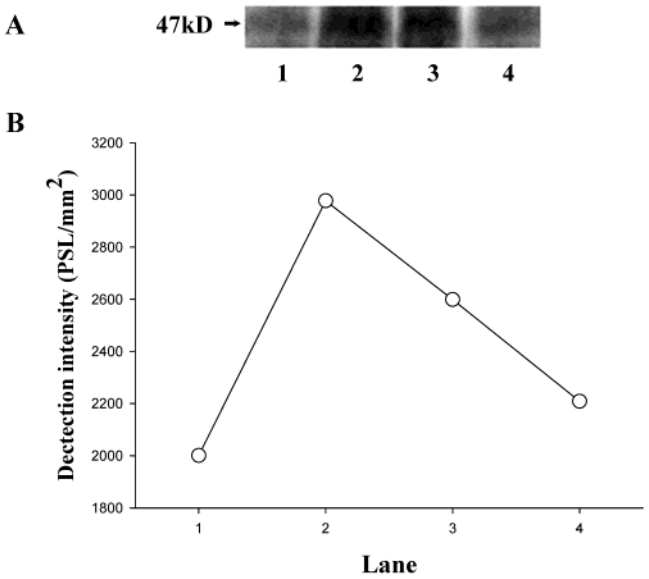

Figure 4. Effect of rutin on phosphorylation of a protein of M_r 47000 (P47) in human platelets challenged with collagen. Platelets were preincubated with rutin (250 and 290 μM) before challenge with collagen (1 μg/mL): (lane 1) platelets with Tyrode's solution only (resting group); (lane 2) platelets with collagen (1 μg/mL); (lane 3) platelets with rutin (250 μM); (lane 4) platelets with rutin (290 μM) for 3 min followed by the addition of collagen (1 μg/mL). (**A**) The profiles are representative examples of four similar experiments. The arrow indicates a protein of M_r 47000 (P47). (**B**) The relative detection densities of the radioactive bands are expressed as PSL/mm^2 (PSL, photostimulated luminescence).

formation, compared to that in resting platelets. In the presence of rutin (250 and 290 μM), nitrate production did not significantly increase after incubation with platelets for 3 min (**Table 2**). These results imply that the antiplatelet activity of rutin may not act through stimulation of the NO/cyclic GMP pathway in human platelets.

Effect of Rutin on Collagen-stimulated Phosphorylation of the 47-kDa Protein. Stimulation of platelets with a number of different agonists (i.e., collagen) induces activation of protein kinase C, which then phosphorylates proteins of M_r 40000−47000 in addition to other proteins (*18*). In this study, phosphorylation experiments were performed to examine the role of rutin in the activation of protein kinase C in human platelets. When collagen (1 μg/mL) was added to human platelets prelabeled with $^{32}PO_4$ for 2 min, a protein with an apparent M_r of 47000 (P47) was predominately phosphorylated as compared with resting platelets (**Figure 4**). On the other hand, rutin (250 and 290 μM) concentration-dependently inhibited the phosphorylation of P47 in human platelets stimulated by collagen (**Figure 4**). In this study, the extent of radioactivity in P47 was expressed as a relative detection density (PSL/mm^2) of the radioactive bands (**Figure 4B**).

DISCUSSION

The results obtained from this study demonstrate that rutin provides antiplatelet activity. The principal objective of this study was to describe the detailed mechanisms involved in the inhibition of agonist-induced human platelet aggregation by rutin. This inhibitory effect of rutin was demonstrable with the use of various agonists (i.e., collagen). In this study, platelet aggregation induced by these agonists (i.e., collagen) appeared to be affected in the presence of rutin. Therefore, this partly implies that rutin may affect Ca^{2+} release from intracellular Ca^{2+} storage sites (i.e., dense tubular systems or dense bodies) (**Figure 3**), and this is in accord with the concept that intracellular Ca^{2+} release is responsible for platelet aggregation (*19*).

Although the action mechanisms of various platelet aggregation agonists, such as collagen, thrombin, and arachidonic acid, differ, rutin significantly inhibited platelet aggregation stimulated by all of them. This implies that rutin may block a common step shared by these inducers. These results also indicate that the site of action of rutin is not at the receptor level of individual agonists. Triflavin acts by binding to the glycoprotein IIb/IIIa complex on the platelet surface membrane, resulting in interference with the interaction of fibrinogen with its specific receptor (*12*). In this study, we found that rutin did not significantly affect FITC−triflavin binding to the glycoprotein IIb/IIIa complex, indicating that the antiplatelet activity of rutin is possibly not directly due to interference with the binding of fibrinogen to its specific receptor on the platelet membrane.

Stimulation of platelets by agonists (i.e., collagen) results in phospholipase C-catalyzed hydrolysis of the minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, with concomitant formation of inositol 1,4,5-trisphosphate and diacylglycerol (*20*). There is strong evidence that inositol 1,4,5-trisphosphate induces the release of Ca^{2+} from intracellular stores (*21*). Diacylglycerol activates protein kinase C, inducing protein phosphorylation and a release reaction. Rutin significantly inhibited collagen-induced activation of protein kinase C (**Figure 4**). Furthermore, our study revealed that phosphoinositide breakdown of collagen (1 μg/mL)-activated platelets was significantly inhibited by rutin (250 μM) (data not shown), suggesting that lowering of intracellular Ca^{2+} mobilization by rutin is related to inhibition of phospholipase C. Moreover, TxA_2 is an important mediator of the release reaction and aggregation of platelets (*22*). Collagen-induced TxB_2 formation, a stable metabolite of TxA_2, was markedly inhibited by rutin (250 and 290 μM) (**Table 1**). It has been demonstrated that phosphoinositide breakdown can induce TxA_2 formation via free arachidonic acid release by diglyceride lipase or by endogenous phospholipase A_2 from membrane phospholipids (*23*). Thus, it seems likely that TxB_2 formation plays a role in mediating the inhibitory effect of rutin on human platelets.

The activation of human platelets is inhibited by two intracellular pathways regulated by either cyclic AMP or NO/cyclic GMP (*24*). The importance of cyclic AMP in modulating platelet reactivity is well established (*15*). In addition to inhibiting most platelet responses, elevated levels of cyclic AMP decrease intracellular Ca^{2+} concentration by the uptake of Ca^{2+} into the dense tubular system (*24*) and negatively affect the action of protein kinase C (*24*). Signaling by NO/cyclic GMP somehow interferes with the agonist-stimulated phosphoinositide turnover that creates Ca^{2+}-mobilizing second messengers and protein kinase C (*25*). However, neither cyclic AMP nor NO/cyclic GMP mediated the inhibitory effect of rutin on agonist-induced platelet aggregation.

In conclusion, the most important observations of this study suggest that rutin markedly inhibits agonist-induced human platelet aggregation. This inhibitory effect may possibly involve the following mechanisms: rutin inhibits the activation of phospholipase C, followed by inhibition of protein kinase C activation and thromboxane A_2 formation, thereby leading to inhibition of the phosphorylation of P47 and intracellular Ca^{2+} mobilization, finally resulting in inhibition of platelet aggregation. These findings suggest that rutin may be an effective tool in treating thromboembolic-related disorders.

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