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ROLE OF CYCLIC AMP IN THE INHIBITION OF HUMAN PLATELET AGGREGATION BY QUERCETIN, A FLAVONOID THAT POTENTIATES THE EFFECT OF PROSTACYCLIN*

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Abstract—Quercetin (3,3′,4′,5,7-pentahydroxyflavone) has previously been shown to inhibit cyclic nucleotide phosphodiesterases prepared from various cell homogenates and the function of intact human platelets. We now report that (1) high concentrations of quercetin raise platelet cAMP levels; and (2) quercetin potentiates the inhibitory effect of prostacyclin (PGI_2) on ADP-induced washed human platelet aggregation and the elevation of platelet cAMP levels elicited by PGI_2. These results suggest a role for cAMP in the mechanism of action of quercetin on blood platelets.

The search for compounds that inhibit human platelet functions is justified by the role of platelets in the initiation of thrombosis and in the development of atherosclerosis and its thromboembolic complications.

We have recently reported the effects of 13 natural flavonoid aglycones on the functions of washed human platelets [1]. These compounds inhibited platelet aggregation and secretion of serotonin caused by adenosine-5′-diphosphate (ADP), collagen or thrombin. Flavonoids also inhibit the activity of 3′,5′-cyclic AMP phosphodiesterases (PDE) prepared from different animal tissue homogenates [2–5]. The structure/activity relationships appeared to be similar for inhibition of platelet aggregation and secretion and for inhibition of PDE activity. Thus it was suggested that the inhibition of platelet aggregation and secretion by flavonoids could be mediated by an elevation of platelet cAMP levels [6].

We demonstrate here that quercetin (3,3′,4′,5,7-pentahydroxyflavone) potentiates the anti-aggregatory action of prostacyclin (PGI_2) and the elevation of platelet cAMP levels caused by PGI_2.

MATERIALS AND METHODS

Materials. Adenosine-5′-diphosphate, sodium salt (ADP) and *N*-2-hydroxyethylpiperazine-*N*′-2 ethanesulfonic acid (HEPES) were obtained from Sigma Chemical Co. (St. Louis, MO). Human fibrinogen (grade L) (Kabi, Stockholm, Sweden) was treated with diisopropylfluorophosphate (Sigma Chemical Co. St. Louis, MO) as described previously [7]. Quercetin was prepared by acid hydrolysis of rutin. One g of rutin (Merck, Darmstadt, F.R.G.) was dissolved in 100 ml of methanol–water (1/1) with 10% (v/v) HCl. The mixture was heated at 100° for 1 hr under reflux. After cooling, methanol was evaporated under vacuum. The solution was left 2 hr at 4°, and the precipitate formed isolated on fritted glass, and dried at 100°. The product was purified twice by crystallization in methanol. Final yield was 650 mg of clear yellow product, m.p. 318° with TLC, u.v. and i.r. characteristics identical with reference compounds. Stock solution of quercetin were prepared in dimethylsulfoxide [DMSO (Fluka, Buchs, Switzerland)], at a concn of 0.5–1 M and kept at 4° in the dark.

Prostacyclin sodium salt (a gift from Dr J. Pike, Upjohn, Kalamazoo, MI) was disolved in 0.05 M Tris buffer, pH 9.36 at 4° (Tris–HCl 1.23 g/l, Tris base 5.13 g/l). A 1 mM stock solution was kept at −30° in small aliquots [8]. All other chemicals were of analytical grade.

Preparation of washed human platelets. Blood was collected from a forearm vein of human healthy volunteers who had not taken any medication for 2 weeks. Six vols. of blood were collected into 1 vol. of acid–citrate–dextrose anticoagulant [9] containing sodium heparin (Roche, France) to a final concn of 1 I.U./ml. Twice-washed platelet suspensions were essentially prepared as described [10]. The final resuspending medium (pH 7.35) was Tyrode solution (which contains 2 mM Ca^{2+}, and 1 mM Mg^{2+}) with 0.35% human albumin (Centre de Transfusion Sanguine, Strasbourg, France) and 5 mM HEPES buffer. Apyrase, prepared from potatoes according to the method of Molnar and Lorand [11], was included in the suspending medium at a concn of 1 μl/ml. This preparation of apyrase (3 mg of protein

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per ml) hydrolysed 1.7 nmole ATP per min per μg protein. The platelets were stored at 37° throughout the experiments. Platelet were counted using a Hycell platelet counter (Model HPC 52, Hycell Europa, Amsterdam, the Netherlands).

Platelet aggregation studies. For platelet aggregation studies, the platelet count was adjusted to 300,000/mm^3. Platelet aggregation was studied at 37° with 1100 rpm stirring in 0.5 ml cuvettes using a turbidimetric device (Payton aggregometer, Scarborough, Ontario) [12]. Inhibitors (quercetin 1 μl or PGI_2 5 μl) or their solvent were added with a micro-pipette (Micro-Pettor, SMI, Berkeley, CA) to 0.5 ml platelet suspension and stirred in the presence of fibrinogen (final concn, 0.8 mg/ml) in the aggregometer for 30 sec before addition of ADP (final concn, 5 μM). The extent of aggregation was measured as the maximum increase in light transmission, and percentage of inhibition was calculated by comparison with the curve obtained in the presence of the control solvents.

Platelet cAMP assay. For cAMP assay the platelet count was adjusted to 500,000/mm^3. One-ml samples of platelet suspensions were incubated with the inhibitors (quercetin, PGI_2) or the solvent (respectively DMSO or Tris–HCl buffer 0.05 M, pH = 9.36) at 37° with stirring at 1100 rpm in the aggregometer cuvette. The time of the simultaneous addition of quercetin and PGI_2 was considered to be time zero of incubation. The reaction was stopped by adding cold trichloroacetic acid (TCA) to achieve a final concn of 5%. The sample was then centrifuged in an Eppendorf centrifuge at 12,000 rpm for 1 min. The supernatant was decanted and trichloroacetic acid extracted by 5 washings with 3 vols. of water-saturated ether. cAMP was then measured in the supernatant by the protein binding method of Gilman [13], using a commercial kit containing [^{3}H]cAMP (The Radiochemical Centre, Amersham France, Versailles).

RESULTS

Effect of quercetin and PGI_2 on the aggregation of human platelets by ADP

PGI_2 (0.1–1 nM) inhibited the aggregation of washed human platelets induced by 5 μM ADP in the presence of fibrinogen. The action of PGI_2 (0.1–0.5 nM) was potentiated by quercetin (2–50 μM) in a dose-dependent manner (Table 1). A low concn of quercetin (2 or 10 μM), which alone did not inhibit platelet aggregation, potentiated the inhibitory effect of PGI_2.

Effect of quercetin and PGI_2 on human platelet cAMP levels

Quercetin, at a concn of 50 or 200 μM, did not raise significantly platelet cAMP levels above the control value (2.16 ± 0.07 pmoles/10^8 platelets, mean ± S.E.M. of six experiments) measured in the presence of DMSO. As shown in Table 1 quercetin at a concn of 200 μM completely inhibited the aggregation of platelets induced by 5 μM ADP. When the concn of quercetin was raised to 500 μM, a small, but significant increase in platelet cAMP levels (3.00 ± 0.25 pmoles/10^8 platelets) was observed.

Table 1. Inhibition by quercetin and PGI_2 of washed human platelet aggregation induced by 5 μM ADP

	Aggregation (% inhibition) PGI_2 (nM)				
Quercetin (μM)	0	0.1	0.25	0.5	1
0	0	28	63	73	100
2	0	/	/	82	/
10	0	50	65	93	/
20	18	88	/	100	/
50	21	94	100	/	/
200	100	/	/	/	/

PGI_2 alone raised significantly platelet cAMP levels above the control value. In the presence of 0.5 nM PGI_2, cAMP levels were raised to 3.05 ± 0.30 pmoles/10^8 platelets ($P < 0.01$) and to 4.34 ± 0.15 pmoles/10^8 platelets ($P < 0.001$) with 1 nM PGI_2 (Fig. 1).

The rise in cAMP level caused by these concns of PGI_2 was significantly potentiated, in a dose-dependent manner, by quercetin (50, 200 or 500 μM) (Fig. 1). The platelet cAMP levels were measured in the suspension at time intervals, fr m 15 to 150 sec after addition of the inhibitors or their solvent. The data presented in Fig. 2 show that quercetin potentiated the effect of PGI_2 on the maximal level of cAMP and retarded its breakdown.

DISCUSSION

Recently, we have studied *in vitro* the effects of quercetin on the function of intact washed human platelets [1]. Quercetin inhibited platelet aggregation and secretion of serotonin induced by ADP, collagen or thrombin. These three agents activate human platelets by at least three different mechanisms [14, 15]. Most inhibitors of platelet function will interfere with one mechanism, for example the ADP or the arachidonate pathway [16]. Inhibitors, which raise platelet cAMP levels, either by stimulating membrane adenylate cyclase (for example, PGI_2) or by inhibiting PDE (for example, dipyridamole), are potent inhibitors of platelet aggregation induced by ADP, collagen or thrombin. Quercetin is an inhibitor of PDE of various tissues [2–5]. Thus, we have examined more directly in this paper the role of cAMP in the inhibition of human platelet aggregation by quercetin.

Four criteria have been suggested to support the hypothesis that an agent could act through a change in the intracellular concn of a cyclic nucleotide [17, 18].

(1) It should have a demonstrable effect on the activities of the enzymes involved in the regulation of the level of the cyclic nucleotide. Quercetin does inhibit cAMP-PDE [2–5].

(2) An exogenous cyclic nucleotide should mimic its pharmacological effects. It has been shown that db-cAMP inhibits platelet aggregation [19].

(3) It should, in the case of a PDE inhibitor, potentiate the effects of hormones that elevate cyclic nucleotide levels. PGI_2 is the most potent stimulator of platelet adenylate cyclase and is synthesised by

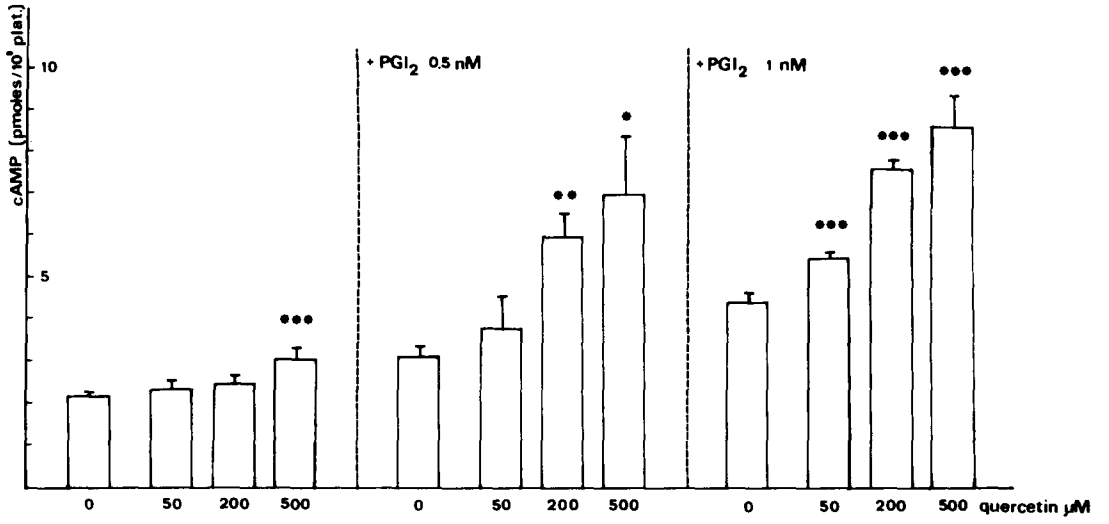

Fig. 1. Influence of quercetin and/or PGI_2 on cAMP levels in suspensions of washed human platelets. cAMP levels measured 1 min after incubation with the inhibitors (quercetin, PGI_2) or the solvent at 37° with stirring at 1100 rpm in the aggregometer cuvette. Values are expressed as means ± S.E.M. (vertical bars) of three experiments. The significance of the difference between cAMP levels in the presence of quercetin and in its absence has been tested using Student's *t*-test: $^*P < 0.05$, $^{**}P < 0.02$ and $^{***}P < 0.01$.

the vessel wall [20]. We have shown here, that a concn of quercetin which potentiated the anti-aggregatory action of PGI_2 (Table 1), potentiated the rise in cAMP level induced by PGI_2 (Fig. 1). Similarly, quercetin potentiated the inhibitory effect of PGE_1 on platelet aggregation induced by ADP, collagen or thrombin and the rise of cAMP induced by PGE_1 [6].

(4) The drug should change the cyclic nucleotide content of intact cells with a dose-dependence and time course consistent with a triggering for response. Quercetin was able to raise cAMP levels in intact platelets, only when it was used at a concn of 500 μM. Using a lower concn of quercetin (50 or 200 μM) we were not able to measure a significant raise in cAMP, although these concns inhibited ADP-induced platelet aggregation. Several other PDE inhibitors, such as dipyridamole [21], BL-3459 and BL-4162 A [22], cilostamide [23] do not raise cAMP levels in platelets at concns that inhibit platelet aggregation. Nevertheless, it is generally admitted that the inhibitory effect of these drugs is mediated by a change in

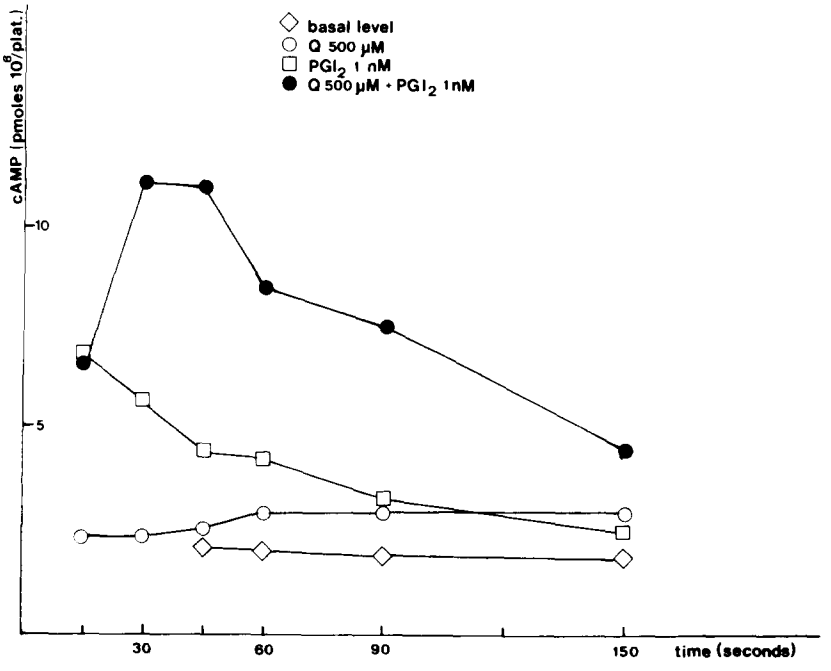

Fig. 2. Influence of time on cAMP levels of washed human platelets exposed to quercetin (Q) and/or PGI_2.

cAMP levels in platelets. At least two possibilities can account for this discrepancy. It is possible that a small change in total cAMP level is sufficient to inhibit platelet aggregation and that our method of measurement of cAMP is not sensitive enough to detect it. The other possibility is that the functional pool of cAMP might only be a small fraction of total platelet cAMP, thus measurements of total cAMP might not reflect changes in the active pool of the cyclic nucleotide [24].

The role of cAMP in mediating some of the effects of quercetin on other cell systems has been studied. Quercetin did raise cAMP levels in Ehrlich ascites tumour cells [25, 26]. In contrast, quercetin inhibited antigen-induced human basophil histamine release without rising cAMP levels in mixed leukocyte suspension [27]. These results add some support to our present findings in platelets that quercetin alone can increase cAMP levels. However, the direct relationship between a rise in cAMP and inhibition of ADP-induced platelet aggregation is best demonstrated in the presence of PGI_2.

In addition to its effect on platelet cAMP, quercetin could inhibit platelet function through a direct effect on Ca^{2+} movements across the plasma membrane. It has been shown that quercetin inhibits Ca^{2+} transport across the membrane of red blood cells [28, 29], the uptake of Ca^{2+} by sarcoplasmic reticulum in muscle fibres [30] and other calcium-dependent cellular responses such as lymphocyte stimulation [31] and the liberation of histamine from mast cells [32, 33].

The complex interaction of platelets with the components of the vessel wall is the first step in thrombosis and atherosclerosis. Quercetin inhibits several aspects of platelet function, raises platelet cAMP levels and potentiates the effect of PGI_2 on platelet aggregation and platelet cAMP levels. Thus, it appears that quercetin and other flavonoids may present pharmacological interest as antithrombotic agents.

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