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MODIFICATION OF PLATELET FUNCTION AND ARACHIDONIC ACID METABOLISM BY BIOFLAVONOIDS

STRUCTURE–ACTIVITY RELATIONS

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Abstract—The mechanism of the antiaggregating activity of flavonoids was studied *in vitro*. The activity of fifteen different compounds was tested on platelet aggregation and arachidonic acid metabolism. The effect of flavonoids on platelet adenosine 3′,5′-cyclic monophosphate (cyclic AMP) levels under basal conditions, as well as after stimulation by prostacyclin (PGI_2), was also measured. The glycons of flavonoids in general and the flavanone derivatives that we tested did not affect platelet function. On the other hand, flavone, chrysin, apigenin and phloretin inhibited platelet aggregation by depressing the cyclooxygenase pathway. In addition, flavone, chrysin and apigenin reduced the platelet cyclic AMP response to PGI_2. This effect was probably mediated by an inhibition of adenylate cyclase. Myricetin and quercetin, however, increased the PGI_2-stimulated rise of platelet cyclic AMP. Both of these flavonoids inhibited primarily lipoxygenase activity. Modification of platelet cyclic AMP metabolism through inhibition of phosphodiesterase activity was found to be the probable mechanism of their antiaggregating effect.

Flavonoids are largely natural compounds widely represented in the human diet [1]. They have complex effects on the metabolism of various cells [2–4]. Some flavonoids produce coronary vasodilation and spasmolysis [5]. Others have been shown to possess anti-asthmatic activity [6], to inhibit mast cell degranulation [7], and to inhibit glycolysis in Ehrlich ascites tumor cells [8]. Inhibition of lipoxygenase activity [9] and leukocyte function [10] has been noted, effects which may explain their anti-inflammatory activity, well known in folk medicine. There is also increasing evidence that flavonoids can affect platelet aggregation [11–13] and adhesion [14]. This is of great interest as it raises the possibility that variations in dietary intake of these compounds could modify platelet responsiveness and thus affect physiological and pathological processes like hemostatis and thrombosis. In a previous study we reported that flavone acted as an antiaggregating agent inhibiting cyclooxygenase activity of human platelets [15]. In this study we report the *in vitro* effect of a large number of different flavonoids on platelet aggregation as well as on adenosine 3′,5′-cyclic monophosphate (cyclic AMP) and arachidonate metabolism.

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MATERIALS AND METHODS

Preparation of platelets and aggregation studies

Platelets were obtained from volunteers selected on the basis of abstention from any medication for 14 days prior to phlebotomy. Platelet rich plasma (PRP) was separated from either citrate–phosphate–dextrose USP (CPD) or citrate-anticoagulated blood (1/10 vol. of 3.8% sodium citrate) at 260 $g \times 15$ min. Aggregation of 0.4 ml PRP was monitored turbidimetrically on an aggregometer (Chrono-Log Corp., Havertown, PA) connected to a recorder. All aggregations were carried out at 37° with PRPs having platelet counts between 2.5 and 3×10^8/ml. Arachidonic acid (AA) was prepared as a 1 mM solution by dissolving sodium arachidonate in degassed 0.14 M NaCl, 10 mM Tris/HCl, pH 7.4 (TBS), containing 0.35% bovine serum albumin (BSA) and 20 μM butylated hydroxytoluene (BHT). Aliquots of this stock solution were frozen and kept at −70° until used. Bovine tendon collagen was solubilized with acetic acid, and dilutions from a stock solution of 5 mg/ml were utilized for measurement of collagen-induced aggregation. All of the compounds tested were prepared as 1 mM solutions in dimethyl sulfoxide (DMSO). Aliquots were frozen and kept at −70° until used. To measure the effect of bioflavonoids on platelet aggregation, PRPs were incubated for 10 min at room temperature with various concentrations of the compounds. The aggregation tracings were then compared to those of controls that were incubated with equivalent amounts of DMSO.

DMSO can depress platelet aggregation and reduce cyclooxygenase activity in these cells [16, 17]. For most experiments the final concentration of DMSO in the incubation mixtures was 0.5%. At that level DMSO had no effect on platelet aggregation or arachidonic acid metabolism. Only at concentrations >2% did this solvent show a dose-dependent inhibition of both functions in platelets.

Evaluation of arachidonic acid metabolism in platelets

Arachidonic acid metabolism was studied by a pulse-labeling technique.

Labeling of platelets. Platelets were washed once with TBS/77 mM EDTA (98:2, v/v) and purified by a modification of the procedure of Hamberg *et al.* [18] in which EDTA and residual plasma were removed by gel filtration on Sepharose CL-2B. Platelets were then counted and adjusted to a concentration of 2×10^8/ml. Flavonoids were tested both as solutions in DMSO (as described for aggregation studies) and as sonicated suspensions. The latter were prepared at a concentration of 1 mM by sonicating the compounds in TBS containing 0.5% BSA and 20 μM BHT. DMSO solutions or sonicated suspensions of the compounds were preincubated for 10 min at room temperature with platelet suspensions. Aggregation was started by addition of 0.5 ml of 30 mM $CaCl_2$ containing 5 N.I.H. units of bovine thrombin and 0.1 μCi of [1-^{14}C]AA (sp. act. 55.8 mCi/mmole; New England Nuclear Corp., Boston, MA) to 4.5 ml of platelet suspension. After 3 min at 37°, the reaction was stopped by addition of an equal volume of 1 N HCl followed by rapid extraction/partition with 10 vol. of chloroform containing 10 μM BHT. The extracts were flash-evaporated under nitrogen *in vacuo* and kept at −70° until analyzed.

Analysis of platelet extracts. Analyses were performed by thin-layer chromatography (TLC) or higher pressure liquid chromatography (HPLC). For the former the extracts were dissolved in 0.2 ml of chloroform and applied to TLC plates coated with hard layer silica gel containing inorganic binding with UV 254 phosphor (Supelco, Bellefonte, PA). After a 45-min development in iso-octane–ethyl acetate–acetic acid–H_2O (50:50:2:2, upper layer), the plates were scanned for radioactivity on a Packard 7220 scanner. Areas of interest on TLC plates were scraped into Aquasol II (New England Nuclear) and counted in a liquid scintillation spectrometer. Arachidonic acid and the AA metabolites of the cyclooxygenase pathway, 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and thromboxane B_2 (TxB_2), as well as of the lipoxygenase pathway, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE), were identified on the basis of their R_f values using appropriate standards.

Arachidonic acid and its conversion products were also separated by HPLC. In these experiments platelet extracts were resuspended in acetonitrile, filtered through 0.2 μm filters (Millipore, Bedford, MA), and analyzed in a Beckman HPLC system (Beckman, Berkeley, CA) consisting of an ultrasphere ODS column (4.5 × 250 mm), a variable wavelength detector, model 165, a dual pump system and an ISCO fraction collector (Instrumentation Specialties Co., Lincoln, NE), model 272. Arachidonic acid and its oxidative conversion products were eluted at a flow rate of 1 ml/min by the following sequence: solvent A 93%, B 7% for 10 min; linear gradient A → 80%, B → 20% in 10 min; linear gradient A → 0%, B → 100% in 20 min. Solvent A contained 30% acetonitrile in water and 0.04% acetic acid; solvent B contained 100% acetonitrile in 0.05% acetic acid. Fractions, 1 ml in volume, were collected and counted using Aquasol II (New England Nuclear) as scintillation fluid. The various metabolites were identified on the basis of their retention times, using appropriate standards as references. In addition, they were characterized pharmacologically by incubating platelets with acetylsalicylic acid and determining the changes resulting from it. Figure 1 shows a typical HPLC separation.

Measurement of cyclic AMP in platelets

Suspensions of control and certain flavonoid-treated platelets were prepared by filtration of PRP through Sepharose CL-2B columns which had been equilibrated with Lindon's buffer [19] containing 0.5% BSA. Platelet suspensions, 1 ml in volume, were incubated at 37° with or without 0.5 nM PGI_2 (final concentration). Aliquots were removed after 1, 15 and 25 min and were precipitated by adding an equal volume of 10% trichloroacetic acid containing [^{3}H]cyclic AMP as internal standard. The precipitates were extracted three times with 5 vol. of diethyl ether. The aqueous phases of the extracts were lyophilized and kept at −70° until the assays could be performed. Cyclic AMP was measured by radioimmunoassay using an [^{125}I]cyclic AMP RIA kit (New England Nuclear) and following the acetylation technique described by Steiner *et al.* [20]. All results were corrected for recovery which was estimated by the internal standard method.

Statistical methods

The statistical evaluation of aggregation studies was performed by using the standard parametric tests, while the significance of the changes in platelet AA metabolism before and after treatment with flavonoids was evaluated by paired *t*-test analysis [21].

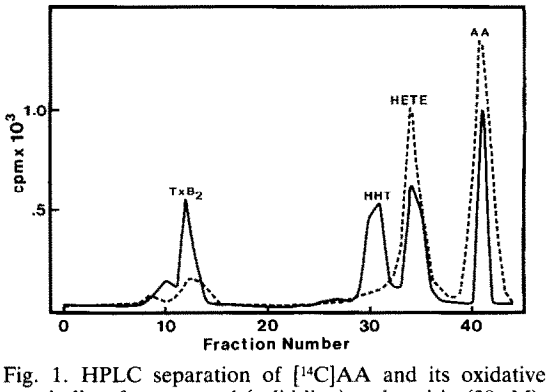

Fig. 1. HPLC separation of [^{14}C]AA and its oxidative metabolites from control (solid line) and aspirin (30 μM)-treated platelets (broken line).

Materials

Solvents and reagents were purchased from the Fisher Scientific Co., Medford, MA. Specialty chemicals were obtained through the Sigma Chemical Co., St. Louis, MO, except for sodium arachidonate (Nu-Chek Prep, Elysian, MN) and bovine thrombin (Parke-Davis, Detroit, MI). Fenwal CPD blood bags were used for larger donations. Sepharose CL-2B was a product of Pharmacia, Piscataway, NJ. Gifts of AA metabolites were gratefully accepted from Dr. John Pike (Upjohn, Kalamazoo, MI).

RESULTS

In Fig. 2 and Table 1 are reported nomenclature and the chemical structures of the bioflavonoids studied.

Aggregation studies

The effects of sixteen different flavonoids on platelet aggregation induced by ADP, AA and collagen are reported in Tables 2 and 3. Flavone, chrysin, flavanone, apigenin, phloretin and kaempferol were the most active inhibitors of platelet response to both AA and collagen. Their inhibition of AA- and collagen-induced aggregation was similar. The I_{50} values (dose of the drug giving 50% inhibition) for collagen ranged betwen 48 and 180 μM and were five to eight times higher than the I_{50} values for AA-induced aggregation. Myricetin and quercetin had a very strong antiaggregating activity against AA but were almost ineffective when collagen was used to induce aggregation. The glycons of quercetin and phloretin, rutin and phlorizin, were found to be without inhibitory activity at doses up to 200 μM. Flavanone required higher doses than flavone to inhibit AA- and collagen-stimulated aggregation but was a more potent inhibitor than its hydroxylated derivatives.

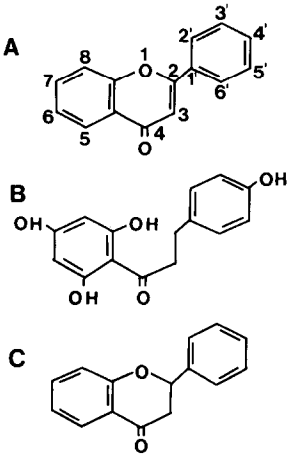

Fig. 2. Chemical structures of flavone (A), phloretin (B), and flavonone (C).

The effects of eight different flavonoids on ADP-induced aggregation were tested by using platelets from six different donors. Myricetin, quercetin and chrysin inhibited both waves of aggregation. Myricetin and quercetin were most effective on the first wave, while chrysin inhibited primarily secondary aggregation. Flavone, apigenin and phloretin suppressed in most cases the second wave of aggregation. Kaempferol showed no significant effect on ADP-induced platelet aggregation.

Platelet cyclic AMP

The effects of eight different flavonoids on platelet cyclic AMP level were examined under basal conditions as well as after stimulation with 0.5 nM PGI_2. None of the flavonoids that we studied caused a significant change in the basal level of platelet cyclic AMP. The PGI_2-induced rise and subsequent decline

Table 1. Nomenclature of the flavonoids studied*

	Substituents†						
Flavonoids	3	5	7	2′	3′	4′	5′
Flavones							
Flavone	H	H	H	H	H	H	H
Apigenin	H	OH	OH	H	H	OH	H
Chrysin	H	OH	OH	H	H	H	H
Kaempferol	OH	OH	OH	H	H	OH	H
Morin	OH	OH	OH	OH	H	OH	H
Myricetin	OH	OH	OH	H	OH	OH	OH
Quercetin	OH	OH	OH	H	OH	OH	H
Rutin	O Rutinose	OH	OH	H	OH	OH	H
Flavanones							
Flavanone	H	H	H	H	H	H	H
Naringenin	H	OH	OH	H	H	OH	H
Naringin	H	OH	O Rhamnose	H	H	OH	H
Hesperetin	H	OH	OH	H	OH	OH	H
Hesperidin	H	OH	O Rhamnose	H	OH	OH	H
Flavanpentols							
Catechin	OH	OH	OH	H	OH	OH	H

* Phloretin and its glycone, phlorizin, which are propiophenone derivatives, are not reported in this table.

† These substituents are for the positions of the structural formulas shown in Fig. 2.

Table 2. Inhibition of AA- and collagen-induced platelet aggregation by flavonoids

Flavonoids	I_{50} (μM) AA (150 μM)	Collagen (5 μg/ml)
Flavone	7.2	48
Chrysin	13.2	70
Phloretin	16	75
Apigenin	18	90
Kaempferol	24	180
Morin	50	>200
Myricetin	24	>200
Quercetin	18	>200
Flavanone	21	58
Catechin	87	>200
Rutin	>200	>200
Naringin	>200	>200
Naringenin	90	>200
Phlorizin	>200	>200
Hesperetin	120	>200
Hesperidin	>200	>200

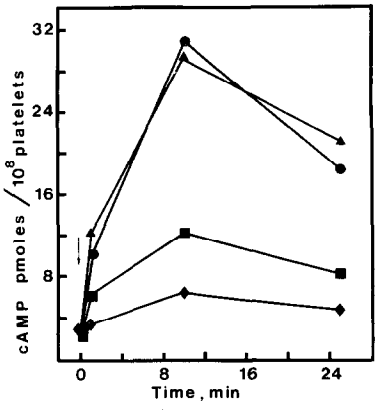

Fig. 3. Effects of flavonone (◆), quercetin (▲) and myricetin (●) on cAMP levels of platelets stimulated with 0.5 nM PGI_2. Cyclic AMP was measured by RIA as described under Materials and Methods. The concentration of flavonoids was 50 μM. A DMSO control (■) is also shown. The arrow indicates the addition of PGI_2. Each point represents the mean of two measurements.

of cyclic AMP were essentially similar in control, kaempferol- or phloretin-treated platelets. Quercetin, myricetin and to a lesser degree morin enhanced the PGI_2-stimulated increase of platelet cyclic AMP. Markedly elevated values persisted even 25 min after stimulation (Fig. 3). Flavone, apigenin and chrysin reduced the response of platelets to PGI_2. Cyclic AMP values were approximately one-half of the controls at each sampling time.

Oxidative conversion of arachidonic acid

While for most flavonoids the difference in the inhibitory effect between sonicated and DMSO-solubilized preparations was insignificant, some clearly were more active when added as DMSO solutions (see Fig. 4). Thereafter, flavonoids were always tested as DMSO solutions. [^{14}C]AA metabolites were routinely analyzed by TLC, but the most effective flavonoids were further studied by HPLC. The results obtained are reported in Table 4. At a concentration of 50 μM, most flavonoids including flavone, chrysin, phloretin, flavanone, apigenin and kaempferol (in order of decreasing potency) inhibited cyclooxygenase activity. Kaempferol, in addition, reduced lipoxygenase activity slightly.

Myricetin and quercetin, when tested at a concentration of 10 μM, exerted strong inhibition of the lipoxygenase pathway. At doses of 50 μM the conversion of AA into both pathways was potently suppressed. Morin, which is chemically closely related to the two above-mentioned compounds, was found to be a much weaker lipoxygenase inhibitor. As observed for AA- and collagen-induced aggregation, the glycones and the flavanone derivatives were found to have no significant inhibitory activity.

DISCUSSION

Our studies have shown that many of the flavonoids are capable of modifying platelet AA metabolism. Most of the compounds we tested inhibited cyclooxygenase activity. Myricetin and quercetin, however, blocked both cyclooxygenase and lipoxygenase pathways at high concentrations. At low con-

Table 3. Inhibition of ADP-induced aggregation by flavonoids*

Flavonoids	Character	Maximal aggr. (%)	Slope 1° wave
None	1° and 2° wave	58.8 ± 6.9	70.8 ± 7.6
Flavone	1° wave only	38.0 ± 5.8 $P < 0.005$	69.5 ± 7.3 NS
Myricetin	1° and 2° wave	45.0 ± 4.6 $P < 0.001$	52.8 ± 3.1 $P < 0.0005$
Apigenin	1° and 2° wave in 4, 1° wave only in 2	49.0 ± 9.6 $P < 0.5$	68.7 ± 5.1 NS
Chrysin	1° wave only	43.2 ± 4.9 $P < 0.0005$	63.7 ± 5.3 $P < 0.5$
Kaempferol	1° and 2° wave	56.8 ± 5.3 NS	70.8 ± 7.5 NS
Quercetin	1° and 2° wave	58.2 ± 5.8 NS	57.0 ± 3.0 $P < 0.005$
Morin	1° and 2° wave	56.8 ± 4.8 NS	69.8 ± 6.1 NS
Phloretin	1° wave only in 3, 1° and 2° wave in 3	42.8 ± 2.4 $P < 0.0005$	69.7 ± 4.6 NS

* Platelet aggregation was induced with 4 μM ADP. Values are expressed as means ± S.D. of six experiments for each flavonoid and control (no inhibitor). Statistical significance (t-test) is given as a P value; NS = not significant.

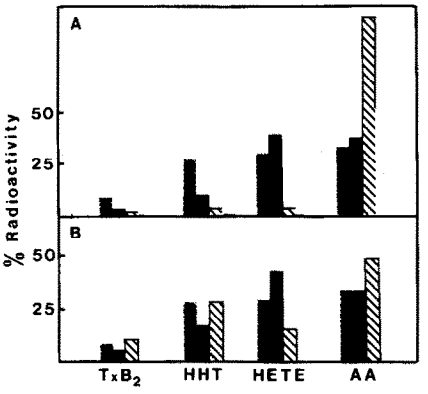

Fig. 4. Effects of flavone and quercetin on AA metabolism. Distribution of [^{14}C]AA and its metabolites in control (narrow striped), flavone- (solid black), and quercetin- (wide striped) treated platelets is shown. The compounds were tested at a concentration of 50 μM (A) and 10 μM (B). The shaded bars in (A) show the response of platelets pretreated with 50 μM quercetin which was added as a sonicated suspension. The bars represent values of a typical experiment. Three were performed. The distribution patterns of [^{14}C]AA for the individual experimental and control preparations were identical in all three experiments.

centrations lipoxygenase was the primary target of inhibition. In previous investigations on the effect of flavonoids on cyclo- and lipoxygenases, enzyme preparations from plants and tissues [4] or from sonicated rabbit platelets [9] were used. These studies showed that flavonoids were able to inhibit AA metabolism although with very evident differences in the relative potency of the agents. Such differences are probably due to varying sensitivity of the enzymes derived from the diverse sources.

Flavone, parent compound of flavonoids, was found [15] to be a potent selective inhibitor of the cyclooxygenase pathway. It is opportune, therefore, to relate changes of activity to modifications of its basic structure. The following observations can be made: (a) Reduction of the C_2–C_3 double bond results in loss of inhibitory activity on the cyclooxygenase pathway; this is based on a comparison of flavone and flavanone. (b) The hydroxylation of the flavone ring in positions 5 and 7, e.g. chrysin, does not alter significantly the inhibitory activity, but the compounds having one or more hydroxyl groups on the other rings were clearly less effective. (c) The flavonoids with a hydroxyl group in position 3, i.e. morin, quercetin, myricetin and kaempferol, all inhibited lipoxygenase although with different potency and selectivity. (d) Opening of the pyranone ring which changes apigenin to phloretin does not abolish inhibition of the cyclooxygenase pathway. (e) The glycosylated derivatives of the various flavonoids were clearly less effective than their corresponding aglycones, e.g. rutin vs quercetin or phloretin vs phlorizin. This cannot be ascribed to reduce membrane permeability of the glycosylated flavonoids since the same observations have been made with isolated enzyme preparations [4] and sonicated platelets [9].

The observed modifications of platelet AA metabolism by flavonoids, exhibiting variable inhibition of one or both oxygenase pathways, are probably not mediated by an effect on cyclic AMP. Some of the flavonoids that were very effective as antiaggregating agents, e.g. flavone, chrysin and apigenin, strongly reduced PGI_2-induced stimulation of cyclic AMP.

Table 4. Effects of flavonoids on [^{14}C]AA utilization by thrombin aggregated platelets*

Flavonoids	Conc (μM)	Expt No.	TBX_2	HHT	HETE	AA
Apigenin	50	4	0.37 ± 0.11†	0.43 ± 0.08†	1.25 ± 0.11†	1.31 ± 0.1†
Catechin‡	50	2	0.94	0.77	1.09	1.07
Chrysin	50	4	0.20 ± 0.14†	0.26 ± 0.09†	1.22 ± 0.38	1.41 ± 0.15†
Flavone	50	7	0.12 ± 0.1†	0.18 ± 0.14†	1.3 ± 0.2†	1.35 ± 0.2†
Flavanone	50	4	0.34 ± 0.08†	0.41 ± 0.1†	1.28 ± 0.11†	1.21 ± 0.1†
Hesperetin‡	50	2	0.85	0.90	1.23	1.05
Hesperidin‡	50	2	0.98	1.1	1.05	0.96
Kaempferol	50	4	0.41 ± 0.15†	0.39 ± 0.13†	0.80 ± 0.1†	2.10 ± 0.4†
Morin	50	4	0.8 ± 0.09	0.91 ± 0.07	0.77 ± 0.11†	1.48 ± 0.2†
Myricetin	50	4	0.05 ± 0.011†	0.04 ± 0.014†	0.031 ± 0.02†	5.41 ± 0.3†
Myricetin	10	2	1.02	1.2	0.32	2.21
Naringin‡	50	2	0.98	1.01	1.04	0.16
Naringenin‡	50	2	1.01	1.03	0.97	0.96
Phloretin	50	6	0.34 ± 0.07†	0.40 ± 0.09†	1.08 ± 0.11	1.25 ± 0.15
Phlorizin	50	2	0.96	1.03	1.02	0.98
Quercetin	50	6	0.03 ± 0.01†	0.04 ± 0.01†	0.03 ± 0.01†	6.27 ± 0.25†
Quercetin	10	2	0.81	0.52	0.12	4.61
Rutin‡	50	2	1.08	0.95	1.01	1.05

* Each experiment was run with its own control. The radioactivities corresponding to AA and its oxidative conversion products were generally separated by HPLC and counted. Each fraction was expressed as a percentage of the total incorporated radioactivity. The ratios of these fractions over their counterparts derived from control platelets are listed in the four columns on the right of this table. Means ± 1 S.D. are shown for experiments of $N > 2$. For experiments of $N = 2$, only the mean values are listed.

† Statistical significance, $P < 0.001$.

‡ For these experiments, analyses of AA metabolites were performed by TLC.

On the other hand, compounds like myricetin and quercetin enhanced platelet cyclic AMP increases induced by PGI_2. The potentiation of the PGI_2 effect on cyclic AMP levels by quercetin has been described previously [22] and is probably due to an inhibition of phosphodiesterase [23–26]. As suggested by Ferrell *et al.* [24], this inhibition arises from the similarity between the pyranone ring of flavonoids and the pyrimidine ring of adenine. According to this hypothesis, the opposite effects exerted by different flavonoids on the PGI_2-induced cyclic AMP response could be due to preferential inhibition of either phosphodiesterase or adenylate cyclase. These and other enzymes [27–30] inhibited by flavonoids require adenine nucleotide as cofactor. The multiple effects of flavonoids make it difficult to relate their antiaggregating effect to a single pharmacological action.

On the basis of our results it is possible to suggest that inhibition of cyclooxygenase plays a major role in the antiaggregating effect of such flavonoids as flavone, apigenin, chrysin and phloretin. We have found that this inhibition is due to an increase in cyclic AMP. Myricetin and quercetin which inhibit primarily lipoxygenase activity have to be considered in a different light. Although their antiaggregating activity was relatively low, they were able to inhibit the first wave of ADP-induced aggregation and increase the cyclic AMP response to PGI_2.

While the functional consequences of lipoxygenase inhibition are quite definite in white cells, this is not so in platelets. Okuma and Uchino [31] have reported several patients with myeloproliferative syndrome who exhibited distinct inhibition of lipoxygenase while platelet aggregation was enhanced. Shunting of arachidonic acid into the cyclooxygenase pathway was thought to be responsible. In our experiments both quercetin and myricetin, while inhibiting lipoxygenase, failed to enhance platelet aggregation. This is not surprising as there was no or very little increase (only with 10 μM myricetin) in the conversion of arachidonic acid into HHT and TxB_2. Arachidonic acid utilization, in fact, was reduced sharply.

Our results show that a large number of flavonoids possess antiaggregating activity. This antiplatelet effect appears to be mediated through different mechanisms of action.

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