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ON THE MECHANISM OF ANTITHROMBOTIC ACTION OF FLAVONOIDS

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Abstract—Flavonols (quercetin and rutin) and flavanes (cyanidol and meciadonol) were studied for their effect on non-enzymatic lipid peroxidation, lipoxygenase and cyclo-oxygenase activities, binding to albumin and platelet membranes. These biochemical properties of four flavonoids were compared with respect to their antithrombotic action *in vivo* and their efficacy at influencing the platelet–endothelium interaction *in vitro*. All four flavonoids inhibited the ascorbate-stimulated formation of malondialdehyde by boiled rat liver microsomes (quercetin > rutin ≈ cyanidol ≈ meciadonol) and inhibited platelet lipoxygenase activity (quercetin > cyanidol > meciadonol > rutin) whereas only flavonols, but not flavanes, stimulated cyclo-oxygenase and were bound to platelet membranes. The same two flavonols dispersed platelet thrombi which were adhering to the rabbit aortic endothelium *in vitro* (EC_{50} for quercetin was 80 nM and for rutin 500 nM) and prevented platelets from aggregation over blood-superfused collagen strip *in vivo* (ED_{50} for quercetin was 5 nmol/kg and for rutin 33 nmol/kg i.v.). Cyanidol and meciadonol were not effective as anti-thrombotic agents. It is concluded that activated platelets adhering to vascular endothelium generate lipid peroxides and oxygen-free radicals which inhibit endothelial biosynthesis of prostacyclin and destroy endothelium-derived relaxing factor (EDRF). Flavonols are anti-thrombotic because they are selectively bound to mural platelet thrombi and owing to their free radical scavenging properties resuscitate biosynthesis and action of endothelial prostacyclin and EDRF. Thus, flavonols release the thrombolytic and vasoprotective endothelial mediators only in these vascular segments which are covered by a carpet of aggregating platelets.

Flavonoids are weak inhibitors of platelet aggregation in platelet-rich plasma [1–5]. We have recently reported [4, 5] that contrary to their modest potency *in vitro* two flavonols, quercetin and rutin, are powerful anti-platelet and antithrombotic agents *in vivo*.

The effect of flavonoids on the activity of the arachidonic acid cyclo-oxygenase (COX) and lipoxygenase (LOX) in platelet has been reported previously [2–7]. Speculation on the relationship between this biochemical property of flavonoids and their platelet-suppressant action is weakened by the rather ambiguous effect of flavonoids on the COX activity [2, 4, 5, 8–11] and the controversy concerning supposed correlation between the inhibition of LOX and the anti-aggregatory potency of a number of flavonoids [12, 13]. An anti-oxidant or oxygen free-radical scavenging action of flavonoids [10, 14–16] is likely to be responsible for their inhibitory effect on COX [17] or LOX [18] activities in platelets, however, it may well be that the anti-oxidant action of flavonoids *per se* plays a role in their anti-thrombotic action since lipid peroxides [19] and superoxide anions [20] are inhibitors of prostacyclin and "endothelium-derived relaxing factor"—EDRF, both of which have platelet-suppressant action [19, 21]. In line with this assumption we have compared the effect of two flavonols and two flavanes on enzymic and non-enzymic lipid peroxidation, and binding to platelet membranes with their anti-thrombotic potencies.

MATERIALS AND METHODS

Effect on LOX and COX activities. Two lipoxygenases were used: crystalline soybean lipoxygenase and fresh 100,000 *g* supernatant from the horse blood platelet homogenate which contained crude 12-lipoxygenase. The activities of both preparations were measured as μmoles of O_2 consumed in the presence of 100 μM of arachidonic acid as described previously [4, 5, 22, 23]. Oxygen consumption in the presence of flavonoids was compared with that in control samples and expressed as per cent of the inhibition. Regression curves were constructed and IC_{50} values calculated (in the tables *b* values indicate the slope of a regression line, while *r* is the regression coefficient). The activity of COX from ram seminal vesicle microsomes was measured polarographically in the presence of 100 μM of arachidonic acid. From the slope of the oxygen consumption curve the initial reaction velocity was calculated [4, 5].

Anti-oxidant properties. Ascorbate-stimulated oxidation of boiled rat liver microsomes was measured as an amount of malondialdehyde (MDA) formed during the incubation of microsomes in the presence of ascorbic acid (200 μM) during 90 min at 37°. MDA was detected by the thiobarbituric acid method [24].

Binding to albumin and platelet membranes. Binding of flavonoids to bovine serum albumin was determined in terms of displacement of 8-anilino-1-naphthalene sulphonate (ANS) from its binding sites as

previously described [25]. We have also adapted this method to measure binding of flavonoids to platelet membranes. Rabbit platelets washed with prostacyclin were prepared from platelet-rich plasma [26]. Pellets containing these platelets were suspended in distilled water, frozen (−70°) and thawed five times. The resulting lysates were centrifuged for 15 min at 5000 *g*. The supernatant was discarded and pellets suspended in a volume of 0.1 M phosphate buffer pH 7.4 adjusting a suspension of platelet membranes to 6×10^7 platelets/ml. Binding of flavonoids to platelet membranes was measured as follows: 0.1 ml of platelet membrane suspension; 0.1 ml of phosphate buffer pH 7.4; 0.1 ml of solvent or the flavonoid solution and 0.2 ml of 500 μM of ANS solution in 0.1 M phosphate buffer pH 7.4 were incubated for 5 min at the room temperature and the fluorescence was read at 470 nm after excitation at 380 nm (both values uncorrected). The fluorescence of samples containing flavonoids was compared with that of samples containing flavonoid solvents and per cent of quenching of the fluorescence was calculated. IC_{50} values were computed from the regression lines. Concentration of the fluorescence probe (ANS) used in these experiments was found to saturate completely binding sites of ANS in platelet membranes.

Platelet–vascular endothelium interaction in vitro. A tubular segment of fresh rabbit thoracic aorta (RbA), 10 cm long, was turned inside out and the upper part of the RbA was tightened. Its lower end was loaded with a weight of 1 g and its upper end was attached to an auxotonic lever of the heart/smooth muscle Harvard transducer (type 384). The lever was balanced with a counterweight to its neutral position. RbA was freely hung in air and protected from environmental disturbances by a polypropylene jacket. RbA was superfused (1.5 ml/min) with pre-warmed (37°) Tyrode solution for 10 min and, later, with pre-warmed (37°) rabbit citrated blood (trisodium citrate 3.8% : whole rabbit blood = 1 : 9 v/v). The superfusate was discarded. A change in weight of blood-superfused RbA was continuously registered using a pre-scaled Watanabe recorder (paper speed 2.5 mm/min). A gain in weight, as checked by electron microscopy, represented deposition of platelet thrombi on the endothelial surface of RbA. A plateau of approximately 200 mg increase in weight was achieved within 20 min of superfusion. Thereafter, flavonoids which were investigated for their disaggregatory action were infused directly into the stream of superfusing blood. Per cent of the weight loss of RbA indicated the potency of their disaggregatory action. In some experiments RbAs were soaked in solutions of either aspirin (110 μM) or 15-HPETE (150 μM) 20 min prior to being superfused with blood and thereafter treated with flavonoids. In another set of experiments RbAs were pre-treated in the same way with mixture of either 15-HPETE (150 μM) + quercetin (3 μM) or aspirin (110 μM) + quercetin (3 μM). In these last series of experiments a degree of maximal gain in weight of the pretreated RbAs was compared with that of non-treated RbAs following superfusion with blood.

Anti-aggregatory action in vivo. The anti-aggregatory effect of flavonoids in anaesthetized cats was studied using the blood-superfused collagen strip technique [4, 5]. Briefly, a rabbit tendon of Achilles was superfused using heparinized arterial blood (2 ml/min) of an anaesthetized cat. After the superfusion blood was returned to the venous system of an animal. An increase in the tendon weight resulting from the deposition of platelet aggregates was continuously registered during a period of 20 min (control aggregation). The platelets were washed off with saline and flavonoids were injected i.v. Then, blood superfusion was restored for the next 20 min. The ratio between degree of aggregation after the pretreatment with flavonoids to the control sample was considered as an index of the *in vivo* anti-aggregatory activity of a flavonoid.

Materials. The following flavonoids were tested: quercetin (Fluka), rutin (Koch-Light), 3-cyanidol (Zyma; Catergen ex tablets) and meciadonol Zy 15029 (3′,4′,5,7-tetrahydroxy-3-methoxyflavan; Zyma). Quercetin and rutin were dissolved in ethanol and added to tested samples in concentrations not exceeding 2% of ethanol. In the case of these flavonoids control samples contained identical concentrations of ethanol. Cyanidol and meciadonol were dissolved in water. The used reagents were:

Table 1. The effect of flavonols and flavanes on the enzymatic oxidation of arachidonic acid

Flavonoid	Inhibition of 15-LOX (IC_{50})	Inhibition of 12-LOX (IC_{50})	Influence on COX
*Quercetin	13,3 μM, N = 18 *b* = 59, *r* = 0.93	1.3 μM, N = 8 *b* = 35, *r* = 0.94	at 10 μM activation by a factor 5.6, N = 6
*Rutin	1596 μM, N = 20 *b* = 36, *r* = 0.86	113 μM, N = 6 *b* = 45, *r* = 0.89	at 100 μM activation by a factor 8.3, N = 9
(+)-3-Cyanidol	5000 μM, N = 16†	7.9 μM, N = 5 *b* = 45, *r* = 0.99	IC_{50} = 943 μM, N = 10 *b* = 38, *r* = 0.93
Meciadonol	1380 μM, N = 11 *b* = 48, *r* = 0.89	50.6 μM, N = 5 *b* = 35, *r* = 0.93	IC_{50} = 1306 μM, N = 7 *b* = 21, *r* = 0.83

b = slope of the concentration–response curve; *r* = regression coefficient; N = number of experiments.

* Published previously.

† 25–35% inhibition occurred in a concentration-independent manner at concentrations of cyanidol from 1 to 5000 μM.

soybean lipoxygenase (EC 1.13.11.12) (Sigma), arachidonic acid (Sigma; purified by column chromatography), 8-aniline-1-naphthalene sulphonate (ANS; Sigma), aspirin (Polfa). 15-HPETE was prepared from arachidonic acid [22]. Other reagents were commercially available, analytical grade.

Statistics. Values of IC_{50} for the inhibition of LOX, COX, lipid peroxidation and binding to albumin and platelet membranes were calculated from the regression equations where x = log concentration of a flavonoid; y = per cent of its inhibitory effect. The number of experiments (N) was adjusted so as to reach the regression coefficient $r > 0.8$; b was the slope of the regression line. The potency of anti-aggregatory and disaggregatory actions of flavonoids is presented as a mean of their effective doses or concentrations ± SE.

RESULTS

Soybean 15-LOX and platelet 12-LOX were effectively inhibited by quercetin (IC_{50} 13 μM and 1.3 μM, respectively). Rutin had a weak inhibitory action on 15-LOX and a stronger action on 12-LOX (IC_{50} = 113 μM). Cyanidol and meciadonol at concentrations of up to 1000 μM hardly influenced 15-LOX activity (Table 1), although they were stronger inhibitors of 12-LOX than rutin. COX from ram seminal vesicle microsomes was stimulated by quercetin and rutin (10–100 μM) and weakly inhibited by cyanidol and meciadonol (100 μM) (Table 1).

All four flavonoids prevented non-enzymic lipid oxidation in rat liver microsomes. Again, the most potent one was quercetin (IC_{50} = 9.4 μM) whereas the remaining three flavonoids were at least six times less potent (Table 2). Flavonols, but not flavanes, displaced a fluorescent probe from hydrophobic sites of albumin and platelet membranes being more potent in this last preparation—by a factor of three—as compared to albumin (Table 3).

In the extracorporeal circulation of anaesthetized cats quercetin and rutin when administered i.v. inhibited the deposition of platelet thrombi on the blood-superfused collagen strip with ID_{50} of 5 ± 0.1 nmol/kg(N = 15) and 33 ± 0.8 nmol/kg (N = 12), respectively. The calculated effective peak plasma levels of these flavonoids were approximately 0.05 and 0.03 μM. Cyanidol and meciadonol at a dose of 1000 nmol/kg i.v. had no anti-aggregatory action. In the same experimental model the reference compound—aspirin—inhibited platelet aggregation by 50% at a dose of 38,000 ± 260 nmol/kg, N = 9.

In our new experimental model for studying the platelet–endothelium interaction quercetin or rutin—when infused into the stream of blood which superfused the endothelial surface of RbA—caused the disaggregation of pre-formed platelet thrombi. EC_{50} for quercetin was 0.08 ± 0.02 μM, N = 20, and for rutin it was 0.5 ± 0.07 μM, N = 10. Cyanidol (10 μM) and meciadonol (10 μM) had no disaggregatory effects. The pre-treatment of RbA with a COX inhibitor (aspirin) or a prostacyclin synthetase inhibitor (15-HPETE) doubled the deposition of platelet thrombi as compared to the control RbA (Fig. 1). When administered over the aspirin or 15-

Table 2. The effect of flavonoids on the non-enzymatic lipid oxidation

Compound	IC_{50} (μM)	b	N
Quercetin	9.4	98.7	17
Rutin	71.9	128.7	5
Cyanidol	64.3	34.4	6
Meciadonol	60.7	33	6

A suspension of boiled rat liver microsomes was incubated (37°) in phosphate buffer in the presence of ascorbic acid at a concentration of 200 μM. After 90 min the suspension was acidified with trichloracetic acid and malondialdehyde (MDA) [24] formed was estimated by thiobarbituric acid method. The control generation of MDA was compared with that in the presence of various concentrations of flavonoids. IC_{50} values and slope of the regression line (b) were calculated from the equation of regression (x = log concentration of a flavonoid; y = percent of inhibiton of MDA generation). Regression coefficients r were greater than 0.8.

Table 3. Binding of flavonoids to albumin and platelet membranes

Flavonoid	IC_{50} for displacement of 1-anilino-8-naphthalene sulfonate from Albumin	b	N	Platelet membranes	b	N
Quercetin	60 μM	66	6	19 μM	28	7
Rutin	626 μM	60	4	196 μM	39	6
Cyanidol	no binding at 3000 μM		2	no binding at 3000 μM		3
Meciadonol	no binding at 3000 μM		2	weak binding (25%) at 3000 μM		3

Albumin or platelet membranes were incubated at the room temperature with 1-aniline-8-naphthalene sulfonate and fluorescence was read at 470 nm after excitation at 380 nm. Per cent of quenching of fluorescence was calculated and the regression equations constructed as described in the footnote to Table 2.

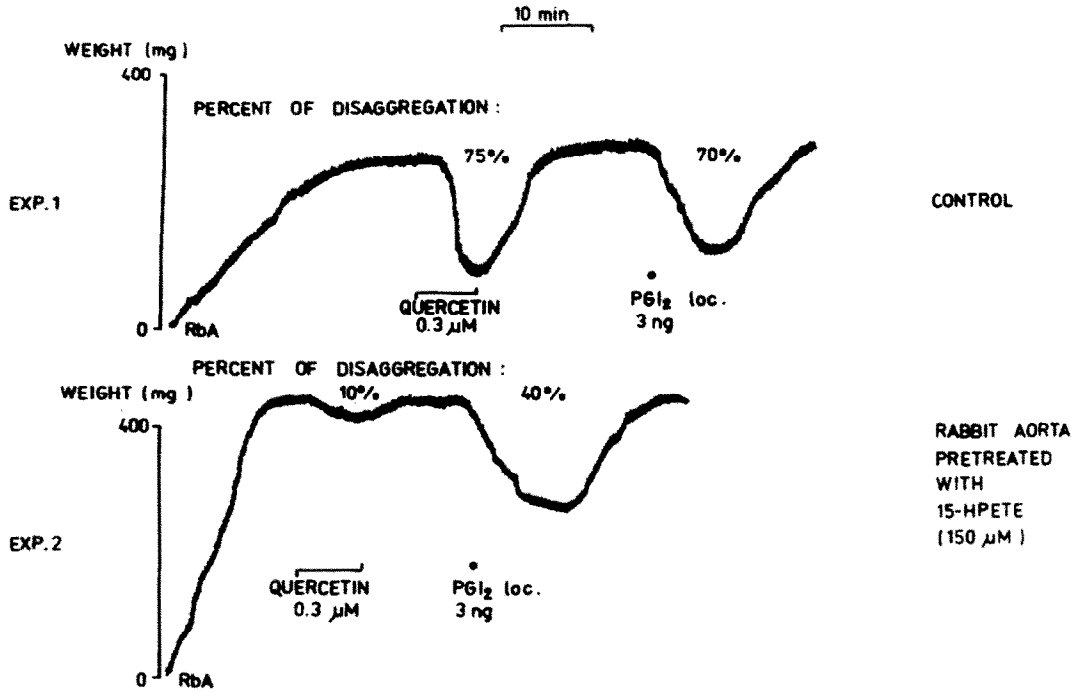

Fig. 1. The disaggregatory effect of quercetin (0.3 μM) on platelet clumps which were adhering to the endothelium of the control (exp. 1) and the 15-HPETE (150 μM)-pretreated (exp. 2) segment of blood-superfused rabbit aorta. In exp. 2 the weight of the deposited platelet clots was doubled in comparison with the control experiment and quercetin—but not PGI_2—lost most of its disaggregatory action.

HPETE pre-treated RbA, both quercetin (0.3 μM) and rutin (2 μM) nearly lost their disaggregatory properties (Table 4). Combined pre-treatment of RbA with 15-HPETE and quercetin abolished the potentiating effect of 15-HPETE on platelet deposition over RbA. On the other hand, combined pre-treatment of RbA with aspirin and quercetin did not prevent an aspirin-induced enhancement of thrombogenic properties of RbA.

DISCUSSION

We have reported [4, 5] that contrary to their modest anti-platelet potency *in vitro* [1–5] quercetin and rutin are potent anti-aggregatory agents *in vivo*. We have shown that unlike these two flavonols, the two flavanes investigated (cyanidol and meciadonol) are deprived of any anti-aggregatory action *in vivo*, moreover, flavonols—but not flavanes—disaggregated platelet thrombi which were adhering to the blood superfused endothelial surface *in vitro*. This disaggregatory action of flavonols was attenuated in the prostacyclin-deprived blood-superfused endothelium of rabbit aortic segments which had been pre-treated with either aspirin or a lipid peroxide (15-HPETE). As expected, the inhibition of the endothelial cyclo-oxygenase (by aspirin) or prostacyclin synthetase (by 15-HPETE) was followed by an increase in thrombogenic properties of the endothelium. In other words, the aortic segments pre-treated with aspirin and 15-HEPE—when superfused with heparinized blood—behaved like collagen strips [4, 5] or aortic preparations from atherosclerotic rabbits [27], i.e. they were highly thrombogenic and

Table 4. The effect of the aspirin and 15-HPETE pretreatment of blood superfused rabbit aortas on the disaggregatory potency of quercetin and rutin

	Percent of maximal disaggregation by	
Pre-treatment	Quercetin 0.3 μM	Rutin 2 μM
None	85 ± 5 N = 12	79 ± 5 N = 3
Aspirin 110 μM	8 ± 5 N = 6	11 ± 4 N = 3
15-HPETE 150 μM	3 ± 6 N = 6	6 ± 3 N = 3

flavonols demonstrated loss of their disaggregatory potency in these preparations. The biochemical injury to the endothelium by 15-HPETE (with a subsequent increase in its thrombogenic properties) did not occur when aortic segments were incubated with quercetin and 15-HPETE concomitantly. Quercetin did not offer this protection against the aspirin pre-treatment.

The above findings strongly suggested that the antithrombotic properties of flavonols *in vivo* might be related to their interaction with the formation of lipid peroxides under a carpet of platelets aggregating over vascular endothelium.

Indeed, flavonols had the required anti-oxidant properties for such action, however, flavanes—which had no anti-thrombotic properties—also inhibited non-enzymatic and enzymatic (12-lipoxygenase) lipid peroxidation to an extent not smaller than that found for rutin. Therefore, the anti-oxidant activity of flavonols *per se* was not a sufficient explanation for their anti-thrombotic action. A major difference between flavonols and flavanes which we found was that the first, but not the latter, were strongly bound to proteins—especially to those in platelet membranes. It is not just a coincidence that flavanes, which are avidly bound to hepatocytes and hardly influence the interaction between platelets and vascular endothelium [29, 30, this paper], are also clinically administered as hepatoprotective but not as vasoprotective agents. Therefore, our hypothesis is that anti-thrombotic and vasoprotective action of quercetin, rutin and other flavonols [5] depends on the combination of two properties in one molecule, i.e. the anti-oxidant or free radical scavenging action and the ability to bind to platelet membranes. Flavonols, being bound and concentrated in platelet membranes, are supposed to prevent the enzymic and non-enzymic generation of lipid peroxides which are formed by activated platelets [3, 31]. We have shown that anti-thrombotic action of flavonols occurs during the interaction of activated platelet with vascular endothelium. Endothelial cyclo-oxygenase and prostacyclin synthetase both are susceptible to the inhibition by lipid peroxides at concentrations higher than 1 μM [19, 32]. It has been repeatedly suggested that such an insult to the secretory function of endothelium initiates thrombogenesis and atherogenesis [34]. Moreover, the second component of the endothelial secretory mechansim, i.e. "endothelium-derived relaxing factor" (EDRF) is avidly destroyed by superoxide anions [20] which are generated by activated platelets along with lipid peroxides [34]. Like prostacyclin, EDRF inhibits platelet aggregation in addition to its vasodilatory activity [21]. Flavonols, by scavenging platelet-derived free radicals, are likely to protect EDRF from destruction by superoxide anions.

We therefore postulate that flavonols enhance local generation of prostacyclin and prolong the half-life of EDRF only in those segments of the vascular endothelium which are covered with platelet thrombi and that is the mechanism of anti-thrombotic and vasoprotective actions of flavonols. Unlike flavanes, flavonols stimulate the cyclo-oxygenase activity in the presence of the substrate at high concentrations (100 μM). This biochemical effect of flavonols might present an additional factor which helps in resuscitation of the platelet-injured endothelial cyclo-oxygenase.

In conclusion, we believe that these flavonoids which (a) are bound to platelet membranes, (b) scavenge free radicals and inhibit lipid peroxidation and (c) stimulate cyclo-oxygenase, are the candidates for vasoprotective and anti-thrombotic drugs.

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