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Effects of flavonoids and transitional metal cations on antigen-induced histamine release from human basophils*

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The flavonoids comprise a large group of naturally occurring compounds widely distributed in the plant kingdom [1, 2]. Various activities have been ascribed to some of the substances including the following: vitamin-C sparing [3–8], epinephrine sparing [5, 8–11], antiinflammatory [8, 11–13], antiviral [13–19], antiallergic [20–23], and mutagenic [24–28]. They are reported to affect the activities of many enzyme systems including ascorbic acid oxidase [7], hyaluronidase [29], catechol-*O*-methyltransferase [30–34], cyclic nucleotide phosphodiesterases [35–37], ATPases [38–43], prostaglandin synthetase [44], lipoxygenase [45], aldose reductase [46, 47], and histidine decarboxylase [48], amongst several others. Quercetin increases cyclic AMP content in Ehrlich ascites tumor cells [49]. The average daily diet contains approximately 1 g of mixed flavonoids 1 and, although the flavonoids are subject to metabolic degradation [50, 51], it is conceivable that pharmacologically active concentrations of these compounds might be achieved under normal conditions and, thus, affect immunologic and other cell functions. At present, the flavonoids are considered to be secondary non-essential dietary cofactors.

Specific effects of certain flavonoids in mammalian cell systems have been demonstrated. For example, quercetin, myricetin, fisetin, and kaempferol inhibit antigen- and mitogen-induced release of histamine from rat mast cells [43, 52]; the stimulated release of beta-glucuronidase from rabbit polymorphonuclear leukocytes is also inhibited by certain flavonoids [53, 54]; other investigations suggest that some flavonoids inhibit lymphocyte glucose uptake [55] and blastogenesis stimulated by phytohemagglutinin [16]. In addition, Schwartz *et al.* [56] have shown that quercetin caused a concentration-dependent inhibition of cytotoxic lymphocyte (CTL) generation in mouse allogenic mixed leukocyte culture and also inhibited the effect of the CTL on their histoincompatible target, ^{51}Cr-labeled P815 mouse mastocytoma cells. Furthermore, quercetin inhibits concanavalin A-induced DNA synthesis in mouse spleen cell cultures [56].

We have also demonstrated [57] that quercetin (but not rutin, the 3-*O*-rhamnosylglucoside of quercetin) is an effective inhibitor of ragweed antigen-induced histamine release from basophils of subjects with hay fever (an *in vitro* model of human IgE- dependent allergic reactions). The inhibition is detectable at micromolar concentrations, is instantaneous in onset of action, is partially reversed by increased buffer calcium concentrations, and is not potentiated by theophylline. The inhibitory effect of quercetin is antagonistic to the histamine release-augmenting effect of heavy water (D_2O). Non-antigen-stimulated basophils are not irreversibly affected by quercetin (washing experiments), and quercetin but not rutin was active as an inhibitor in both the first and second stages of histamine release [58], i.e. both the antigen-dependent, calcium-independent and the antigen-independent, calcium-dependent stages of histamine release were inhibited by quercetin. These observations suggest that only antigen-activated basophils are affected by quercetin. Also, quercetin and several other active flavonoids did not stimulate the synthesis of cyclic AMP in mixed leukocyte preparations.

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In light of the above findings, it seemed of interest to study the effects of other naturally occurring flavonoids on antigen-induced histamine release from human basophils to establish some structure–activity relationships.

Materials and methods

Chemicals. The following flavonoids with noted supply source were utilized in our experiments: flavone, apigenin, chrysin, kaempferol, morin, naringenin, hesperetin, taxifolin, phloretin, catechin, naringin, and neohesperidin dihydrochalcone from the Sigma Chemical Co., St. Louis, MO; quercetin, fisetin, myricetin, flavanone and rutin from the Aldrich Chemical Co., Milwaukee, WI; and galangin and cyanin chloride from Tridom Fluka, Hauppauge, NY. Tangeretin, nobiletin, and hesperidin were donated by Dr. John Attaway, Department of Citrus, State of Florida, Lakeland, FL. All compounds were dissolved in dimethylsulfoxide (DMSO) and were diluted in Tris buffer (25 mM) containing calcium (0.6 mM), magnesium (1.0 mM) and 0.03% human serum albumin (Tris–ACM) [59].

Preparations of leukocyte suspensions. Leukocyte suspensions essentially free of erythrocytes were prepared from blood of subjects with ragweed hay fever (determined by history and positive skin tests) according to method of May *et al.* [60]. The concentration of DMSO in the final leukocyte suspension was 1.0% This concentration of DMSO did not interfere with the analytical technique for histamine or with antigen-induced histamine release. An aqueous extract of whole ragweed pollen was used to activate histamine release.

Measurement of histamine. The spectrophotoflurometric method [61] as modified [60] was used for the determination of histamine. Total histamine was measured in untreated leukocyte suspensions, and histamine content of leukocyte suspensions and of supernatant fractions after different experimental manipulations was determined and the results were expressed as percent of total histamine release. None of the flavonoids studied interfered with the analytical technique for histamine.

Results and discussion

Table 1 shows the effects of various flavonoids on antigen-induced histamine release from human basophils. Quercetin was always the most effective inhibitor and so the ratio of inhibitory activity of all other compounds to quercetin (set at 1.00) was determined for the concentrations studied. It is evident that the parent compounds, flavone and flavanone, lacked activity. Catechin, the flavan congener of quercetin which lacks the C4-keto group, was also inactive. Thus, compounds with the C4-keto but without A or B ring hydroxyls or compounds with A and B ring hydroxyls but without a C4-keto group represent inactive structures. Catechin, however, contains a reduced C2-3 bond and, therefore, is structurally similar to flavanone and the flavanonols which were inactive except for slight activity exhibited by hesperidin and hesperitin. The differential activity comparing a flavonol and a flavanonol is strikingly illustrated in the comparison of quercetin and taxifolin (dihydroquercetin) which differ only in the state of oxidation of the C2–3 bond. Taxifolin was completely inactive, suggesting that the planarity of the gamma-pyrone ring system is important in determining inhibitory activity. The flavylium compound, cyanin chloride, which is closely related to cathechin, was also inactive. The importance of the position of A and B ring hydroxyl groups is noteworthy.

Table 1. Effects of different flavonoids on antigen-induced human basophil histamine release: structure–activity relationships*

Groups and trivial name	Chemical name	Percent inhibition of histamine release (± S.D.) 20 μM	50 μM	Ratio of activity of flavonoid to quercetin (=1.00) at 20 μM	50 μM
Aglycones					
Flavones					
Flavone	2-Phenylchromone	4.2 ± 9.4 (4)†	1.0 ± 7.7 (4)	0.06	0.01
Apigenin	5,7,4′-Trihydroxyflavone	66.5 ± 11.4 (4)	89.4 ± 5.3 (4)	0.78	0.92
Chrysin	5,7-Dihydroxyflavone	7.9 ± 10.1 (5)	17.6 ± 7.6 (5)	0.11	0.18
Tangeretin	5,6,7,8,4′-Pentamethoxyflavone	14.5 ± 12.0 (10)	23.2 ± 18.4 (10)	0.18	0.24
Nobiletin	5,6,7,8,3′,4′-Hexamethoxyflavone	24.2 ± 7.3 (4)	37.9 ± 12.3 (4)	0.28	0.39
Flavonols					
Quercetin	3,5,7,3′,4′-Pentahydroxyflavone	76.3 ± 15.3 (49)	95.8 ± 6.4 (49)	1.00	1.00
Myricetin	3,5,7,3′,4′,5′-Hexahydroxyflavone	48.4 ± 18.9 (6)	77.3 ± 14.5 (6)	0.64	0.82
Fisetin	3,7,3′,4′-Tetrahydroxyflavone	36.1 ± 22.8 (6)	70.9 ± 14.9 (6)	0.47	0.78
Kaempferol	3,5,7,4′-Tetrahydroxyflavone	9.9 ± 14.7 (6)	34.3 ± 15.4 (6)	0.12	0.37
Morin	3,5,7,2′,4′-Pentahydroxyflavone	1.2 ± 11.1 (4)	6.6 ± 13.5 (4)	0.01	0.07
Galangin	3,5,7-Trihydroxyflavone	−3.4 ± 9.5 (3)	−1.5 ± 16.3 (3)	0.00	0.00
Flavanones					
Flavanone	2-Phenylchromanone	−2.7 ± 5.4 (4)	−0.4 ± 7.4 (4)	0.00	0.00
Naringenin	5,7,4′-Trihydroxyflavanone	12.6 ± 12.3 (4)	16.3 ± 15.2 (4)	0.15	0.16
Hesperetin	5,7,3′-Trihydroxy-4′-methoxyflavanone	2.4 ± 11.5 (7)	17.9 ± 12.9 (7)	0.04	0.20
Flavanonols					
Taxifolin	3,5,7,3′,4′-Pentahydroxyflavanone	−1.3 ± 6.1 (4)	8.0 ± 5.9 (4)	0.00	0.08
Dihydrochalcones	β-(*p*-Hydroxyphenyl)2,4,6-trihydroxy				
Phloretin	propiophenone	23.7 ± 14.3 (6)	32.3 ± 27.0 (6)	0.27	0.32
Flavans					
Cathechin	3,5,7,3′,4′-Pentahydroxyflavan	2.8 ± 6.9 (3)	5.7 ± 7.7 (3)	0.03	0.06
Glycosides					
Flavonol glycosides					
Rutin	5,7,3′,4′-Tetrahydroxyflavone-3-*O*-rhamnosylglucoside	−7.8 ± 6.2 (5)	6.7 ± 8.7 (5)	0.00	0.07
Flavanone glycosides					
Hesperidin	5,3′-Dihydroxy-4′-methoxyflavanone-7-*O*-rhamnosylglucoside	22.5 ± 7.3 (3)	27.1 ± 8.3‡ (3)	0.27	0.27§
Naringin	5,4′-Dihydroxyflavanone-7-*O*-rhamnosyglucoside	−1.1 ± 17.3 (3)	14.1 ± 3.6 (3)	0.00	0.14
Dihydrochalcone					
Neohesperidin dihydrochalcone	3,5-Dihydroxy-4-(3-hydroxy-4-methoxy hydrocinnamoyl)phenyl-2-*O*-mannosylglucoside	5.7 ± 0.1 (3)	13.3 ± 7.5 (3)	0.06	0.13

Table 1. *(continued)*

Groups and trivial name	Chemical name	Percent inhibition of histamine release (± S.D.)		Ratio of activity of flavonoid to quercetin (=1.00) at	
		20 μM	50 μM	20 μM	50 μM
Flavylium salt *Cyanin chloride*	3,5,7,3′,4′-Pentahydroxyflavylium chloride-3,5-diglucoside	20.0 ± 2.8 (3)	27.0 ± 12.7§ (3)	0.23	0.27

* Leukocyte suspensions of subjects with documented ragweed hay fever (history and positive prick skin tests) were prepared by dextran flotation [60] and suspended in Tris buffer containing albumin, calcium, and magnesium (Tris–ACM) [59]. The flavonoids were dissolved in dimethylsulfoxide (DMSO; final concentration in reaction mixture was 1%) and an aqueous ragweed extract was employed to induce histamine release. Histamine was measured by the spectrophotofluorometric technique [61]. The flavonoids were routinely added to cell suspensions for a 10-min preincubation at 37° following which antigen was added and the incubation continued for 40 min. Following centrifugation, the supernatant histamine content was measured. Total available histamine was determined in control tubes and results were finally expressed as percent inhibition of histamine release caused by the different compounds. Since quercetin was always the most effective inhibitor at 20 and 50 μM, the activity of all other compounds was related to quercetin (=1.00).

† Numbers in parentheses are number of experiments.

‡ Twenty-five micromolar hesperidin and cyanin chloride.

§ Calculated from the 25 μM concentration used in inhibition experiments.

Morin, which differs from quercetin only in the position of the B ring hydroxyls (2′, 4′, vs 3′, 4′ respectively) was totally inactive. On the other hand, myrecitin, which possesses an additional B ring hydroxyl (5′) compared to quercetin, was slightly less active than quercetin. Rutin, the 3-*O*-rhamnosylglucoside of quercetin, was inactive, suggesting the requirement of a 3-OH group for activity. However, apigenin, which differs from quercetin only in the absence of the 3 and 3′-hydroxyl, was a very effective inhibitor, indicating the non-essentiality of the C3-OH group and suggesting the possibility that the 3-*O*-rhamnosylglucoside group of rutin may sterically hinder the site(s) of quercetin required for inhibitory activity. The importance of B ring hydroxylation is emphasized by the fact that all of the most active compounds, i.e. the flavonols, possessed 3′, 4′-hydroxyl groups or at least a 4′-hydroxyl. Flavonols lacking B ring hydroxyls (chrysin and galangin) were essentially inactive. The polymethoxylated flavonols tangeretin and nobeletin exhibited slight to moderate activity.

Thus, flavone (but not flavanone) derivatives with 4′ or 3′, 4′hydroxyls *and* with the C4-keto-5-OH couplet were most active, and activity was enhanced by the additional presence of the C3-OH group. This pattern of hydroxylation permits chelate formation. Indeed the vitamin C-sparing activity of some flavonoids is related to their ability to chelate Cu^{2+}, an essential co-factor in ascorbic acid oxidation [7, 62]. Therefore, we tested the activities of several divalent metal cations (Cu^{2+}, Co^{2+}, and Mn^{2+}) in blocking the inhibitory activity of quercetin and several other flavonoids on antigen-induced basophil histamine release.

The results shown in Table 2 indicate that Cu^{2+}, Co^{2+}, and Mn^{2+} blocked the inhibitory action of quercetin and other flavonoids in a concentration-dependent manner. Somewhat higher concentrations of the metal cations were required to block quercetin-induced inhibition of histamine release at the highest concentration (50 μM). At quercetin concentrations of 10 and 20 μM, approximately 10 μM divalent transitional metal cation effectively ablated the flavonoid inhibitory activity on histamine release without by themselves significantly affecting histamine release at the concentrations studied. The relative activities of the metal cations were $Cu^{2+} > Co^{2+} > Mn^{2+}$. These results strongly suggest that a chelate was formed between the transitional metal and quercetin in the fluid phase which effectively prevented quercetin from interacting with basophil cell membranes to inhibit antigen-induced histamine release. It is of interest, however, that two flavonoids active in inhibiting basophil histamine release, apigenin and fisetin, were not blocked by Cu^{2+} (data not shown). We have not directly measured chelate formation between quercetin and Cu^{2+} to test our contention that this is the mechanism by which Cu^{2+} blocked the inhibitory effect of quercetin on histamine release. However, the data of Thompson and Williams [62] indicate that quercetin, amongst several other flavonoids, is a most powerful chelator of Cu^{2+} as determined by potentiometric titration. Therefore, we consider it most likely that the effect of Cu^{2+} in our experiments was via a chelation mechanism.

In three experiments we examined the effect of adding Cu^{2+} (10 μM final concentration) to an ongoing histamine release reaction in the presence of 20 μM quercetin in order to test the reversibility of quercetin-induced inhibition of histamine release. Table 3 shows that addition of Cu^{2+} in the early stages of the reaction decreased the inhibition of histamine release caused by 20 μM quercetin. The results indicate that once quercetin has started to exert its inhibitory effect it cannot be completely reversed by Cu^{2+}.

While the biochemical mechanism by which flavonoids inhibit antigen-induced histamine release remains to be elucidated, it is of interest that quercetin and apigenin inhibit beta-glucuronidase release from human polymor-

Table 2. Effects of transitional metal cations on antigen-induced basophil histamine release in the presence of quercetin*

No. of experiments	Quercetin concn	Percent histamine release			
		Control	1 μM	10 μM	50 μM
		Cu^{2+}			
3	None (control)	42.3	38.4	40.7	38.4
	10 μM	13.2	21.0	41.6	39.9
	20 μM	4.7	6.0	44.5	42.5
	50 μM	0.2	0.4	11.6	37.8
		Co^{2+}			
3	None (control)	66.2	68.1	65.0	60.7
	10 μM	34.0	43.4	59.6	62.2
	20 μM	17.1	25.0	46.5	57.2
	50 μM	0.0	0.8	5.6	32.9
		Mn^{2+}			
3	None (control)	59.1	59.7	58.5	58.9
	10 μM	24.5	32.3	47.8	61.0
	20 μM	11.4	17.2	34.3	54.0
	50 μM	1.8	2.3	14.9	46.8

* Solutions of quercetin (dissolved in DMSO, 1% final concentration in cell suspension) were mixed with various concentrations of the metal cations ($CuSO_4$, $CoSO_4$, $MnCl_2$) dissolved in Tris–ACM buffer followed by addition of cell suspensions for 10 min and then an appropriate concentration of ragweed antigen. After 40 min of incubation at 37°, the cells were removed by centrifugation and supernatant histamine was determined.

Table 3. Effect of adding Cu^{2+} at different times after initiation of antigen-induced histamine release in the presence of quercetin (20 μM)*

Time of addition of Cu^{2+} (min)	Percent inhibition of histamine release by 20 μM quercetin	
	$-Cu^{2+}$†	$+Cu^{2+}$
0	0	0
2	92.9	47.7
5	91.2	57.9
10	85.3	71.5
40	85.5	83.1

* Leukocyte suspensions were preincubated with 20 μM quercetin for 10 min and then antigen was added (time 0). Cu^{2+} was added (100 μl) at the indicated times to provide a final concentration of 10 μM, and incubation was continued until a total of 40 min had elapsed. The tubes were then centrifuged and supernatant histamine was measured. Averaged results of three experiments.

† The histamine release reaction in tubes to which Cu^{2+} was not added was stopped by addition of EDTA (1 mM final concentration) to determine the extent of inhibition by quercetin at the noted time.

phonuclear leukocytes (PMN) stimulated with zymosan-activated serum; quercetin also stimulates phospholipid methylation and inhibits phospholipase A_2 activity in these cells (manuscript submitted for publication). It is conceivable, therefore, that quercetin and other active flavonoids inhibit basophil histamine release by an effect on phospholipid metabolism.

In summary, structure–activity relationship studies have been performed on the inhibition of antigen-induced histamine release from human basophils by various naturally occurring flavonoids. Quercetin was the most active compound. Of the transitional metal ions, Cu^{2+} most effectively blocked the inhibitory activity of quercetin, possibly through a chelation mechanism.

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