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FLAVONOIDS: POTENT INHIBITORS OF ARACHIDONATE 5-LIPOXYGENASE

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Various flavonoids were found to be relatively selective inhibitors of arachidonate 5-lipoxygenase which initiates the biosynthesis of leukotrienes with the activity of slow reacting substance of anaphylaxis. Cirsiliol (3', 4',5-trihydroxy-6,7-dimethoxyflavone) was most potent, and the enzyme partially purified from rat basophilic leukemia cells was inhibited by 97% at a concentration of 10 μM (IC_{50}, about 0.1 μM). 12-Lipoxygenases from bovine platelets and porcine leukocytes were also inhibited but at higher concentrations (IC_{50}, about 1 μM), and fatty acid cyclooxygenase purified from bovine vesicular gland was scarcely affected. The compound at 10 μM suppressed by 99% the immunological release of slow reacting substance of anaphylaxis from passively sensitized guinea pig lung (IC_{50}, about 0.4 μM).

Arachidonate 5-lipoxygenase catalyzes the oxygenation of arachidonic acid at C-5 position to produce 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid which is further transformed to various leukotrienes (1). The biological activity of slow reacting substance of anaphylaxis (SRS-A) is now ascribed predominantly to leukotrienes C_4 and D_4 (1). In view of important roles of leukotrienes as mediators of anaphylactic reactions (1) several synthetic compounds have so far been reported as specific inhibitors of 5-lipoxygenase (2-7). Since certain flavonoids such as rutin (8), baicalein (9) and quercetin (10), were earlier reported to show anti-allergic actions, we examined a variety of flavone derivatives isolated from natural sources, for example, leaves of *Salvia officinalis* and *Sesamum indicum*, and clearly demonstrated by the use of a partially purified enzyme from rat basophilic leuke-

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Abbreviation : SRS-A, slow reacting substance of anaphylaxis.

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mia cells that cirsiliol and several other flavonoids were potent and relatively selective inhibitors of 5-lipoxygenase.

MATERIALS AND METHODS

Materials. Syntheses of various flavone compounds were described previously (11-16). [1-^{14}C]Arachidonic acid (55.4 mCi/mmol) was purchased from New England Nuclear. 5-Hydroxy-6,8,11,14-eicosatetraenoic acid was kindly donated by Dr. M. Hayashi of Ono Pharmaceutical Company, and rat basophilic leukemia cells by Dr. S. Narumiya of Kyoto University. Dulbecco's modified Eagle's medium and fetal calf serum were supplied by Gibco. 8-(6-Aminohexyl)-aminoadenosine 5'-triphosphate (Sigma) was linked to Sepharose 4B.

Preparation and Assay of Enzymes. Arachidonate 5-lipoxygenase was obtained from rat basophilic leukemia cells. The cells were cultured in Dulbecco's modified Eagle's medium, supplemented with fetal calf serum (10%), penicillin (75 units/ml) and streptomycin (50 μg/ml) in a humidified 7% CO_2-in-air atmosphere at 37°C. The cells were harvested, washed once and resuspended in 50 mM potassium phosphate buffer at pH 7.4 containing 10% ethylene glycol and 1 mM EDTA at a density of 2 x 10^7 cells/ml. The cell suspension (20 ml) was sonicated at 20 KHz for 30 sec with an aid of Branson Sonifier model 185. After centrifugation at 105,000 x g for 60 min the supernatant solution (cytosol) was adsorbed to and eluted from a 2 x 6-cm column of Sepharose 4B to which ATP was linked with 1,6-diaminohexane as a spacer. The enzyme was purified about 5-fold over the cytosol fraction to a specific activity of about 40 nmol/min/mg protein. This enzyme preparation produced 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid from arachidonic acid as a major reaction product. Details of the purification and properties of enzyme will be published elsewhere. 12-Lipoxygenases were partially purified from bovine platelets (17) and porcine leukocytes (18). Fatty acid cyclooxygenase was purified from bovine vesicular glands as described previously (19). Lipoxygenases were assayed at 30°C for 5 min in a reaction mixture (0.2 ml) containing 50 mM potassium phosphate buffer at pH 7.4 and 25 μM [1-^{14}C] arachidonic acid (30 nCi). For 5-lipoxygenase assay the mixture was fortified with 2 mM $CaCl_2$ and 2 mM ATP (20). The coefficient of variation of intraassay was 5.2%. Cyclooxygenase was assayed at 24°C for 2 min in a reaction mixture (0.2 ml) containing 0.1 M Tris-HCl buffer at pH 8.0, 2 μM hematin, 5 mM tryptophan and 25 μM [1-^{14}C]arachidonic acid (30 nCi). Preincubation of enzyme with flavonoids was performed for 5 min. Procedures of extraction, separation and quantitation of reaction products were described previously (18-20).

Biosynthesis of SRS-A by Sensitized Guinea Pig Lung. Outbred Hartley male guinea pigs (400-450 g) were passively sensitized by intraperitoneal injection of 2 ml of the antiserum against (benzyl-penicilloyl)$_{29}$-bovine gamma globulin (21). After 2 days the lungs were perfused through the pulmonary artery with Tyrode's solution (20 ml/animal). The isolated lungs were cut into 0.5-mm fragments with a McIlwain tissue chopper. Fragments (400 mg wet weight) were suspended in 3.88 ml of Tyrode's solution. After temperature equilibration at 37°C the reaction mixture was preincubated for 5 min with 20 μl of flavonoid at various concentrations or its vehicle (dimethyl sulfoxide). Antigen solution (100 μl) was added at a final concentration of 1 μg or 10 μg/ml. After incubation at 37°C for 10 min the reaction mixture was centrifuged at 1,700 x g for 30 min at 4°C, and the supernatant solution was stored at -80°C until assay. SRS-A was bioassayed on the isolated guinea pig ileum by the bracket technique in the presence of atropine (50 ng/ml) and mepyramine (100 ng/ml) as described previously (22). Release of histamine was assayed by a fluorometric method (23).

RESULTS AND DISCUSSION

The cytosol fraction of rat basophilic leukemia cells was incubated with [1-^{14}C]arachidonic acid, and the ethereal extract of the reaction mixture was

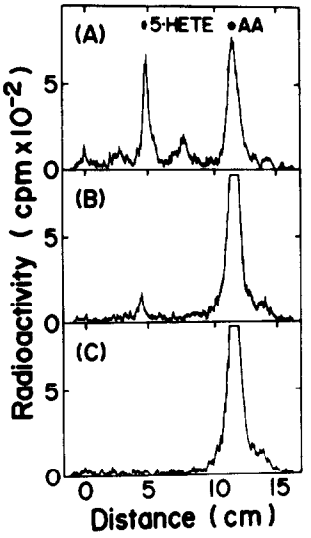
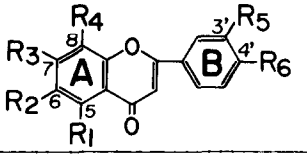


Fig. 1 Inhibition of 5-lipoxygenase by cirsiliol. The cytosol fraction of rat basophilic leukemia cells (110 μg of protein) was incubated in the standard mixture in which cirsiliol was included at 0 μM (A), 1 μM (B), and 10 μM (C). Authentic arachidonic acid (AA) and 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) were also run.

examined by silica gel thin layer chromatography. As shown in Fig. 1A, a major radioactive product comigrated with authentic 5-hydroxy-6,8,11,14-eicosatetraenoic acid, and its identification was confirmed by high performance liquid chromatography, ultraviolet absorption spectrometry and gas chromatography-mass spectrometry. When cirsiliol (3',4',5-trihydroxy-6,7-dimethoxyflavone. See Table I for its structure, F-26) was present at 1 or 10 μM in the reaction mixture, the production of 5-hydroxy acid was markedly suppressed (Figs. 1B and C). As shown in Fig. 2, when cirsiliol was tested with the partially purified enzyme, the 5-lipoxygenase reaction was inhibited in a dose-dependent manner. Almost complete inhibition was observed with 10 μM cirsiliol, and its IC_{50} value was estimated to be about 0.1 μM. Dialysis of the mixture of enzyme and cirsiliol did not reverse the enzyme activity, the result suggesting an irreversible inhibition. Furthermore, Lineweaver-Burk plots showed that the inhibition was of non-competitive type. 12-Lipoxygenases partially purified from bovine platelets and porcine leukocytes were also inhibited by this compound. However, IC_{50} values for these enzymes (about 1 μM) were higher by one order of magnitude than that for 5-lipoxy-

Table I. Effect of various flavonoids on 5-lipoxygenase.



	Substitution						Enzyme activity(%)	
	R_1	R_2	R_3	R_4	R_5	R_6	1 μM	10 μM
F-0 (flavone)	H	H	H	H	H	H	91	77
F-26 (cirsiliol)	OH	OCH_3	OCH_3	H	OH	OH	8	0
F-77	OH	OCH_3	OCH_3	H	H	OH	92	36
F-79	OH	OCH_3	OCH_3	H	OH	OCH_3	89	78
F-75	OH	OCH_3	OCH_3	H	OCH_3	OCH_3	98	92
F-27 (pedalitin)	OH	OH	OCH_3	H	OH	OH	9	0
F-28	OH	OH	OH	H	OH	OH	50	9
F-81	OCH_3	OCH_3	OCH_3	H	OH	OH	33	6
F-2	OH	OCH_3	OCH_3	OCH_3	OH	OH	17	0
F-8 (quercetin)[a]	OH	H	OH	H	OH	OH	70	15
			Catechol				100	96

Reactions of 5-lipoxygenase were carried out in the standard assay mixture in which various flavonoids were added at concentrations indicated. The enzyme activity of 100% corresponded to the arachidonate oxygenation of 1.8 nmol/5 min.

a: A hydroxyl group present at 3-position.

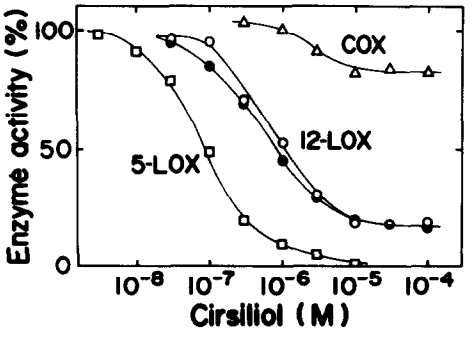


Fig. 2 Inhibitory effect of cirsiliol on various lipoxygenases and fatty acid cyclooxygenase. Enzyme assays were carried out as described in "Materials and Methods" in the presence of varing concentrations of cirsiliol. The enzymes used were 5-lipoxygenase (12 μg of protein) of rat basophilic leukemia cells (□), 12-lipoxygenase (10 μg of protein) of bovine platelets (○), 12-lipoxygenase (3 μg of protein) of porcine leukocytes (●), and cyclooxygenase (3.6 μg of protein) of bovine vesicular gland (△). 5-LOX, 5-lipoxygenase; 12-LOX, 12-lipoxygenase; and COX, cyclooxygenase.

genase. On the other hand, fatty acid cyclooxygenase purified from bovine vesicular gland was only slightly inhibited at higher concentrations of cirsiliol. A 5-lipoxygenase from guinea pig peritoneal leukocytes (20) was also inhibited by cirsiliol with an IC_{50} of about 0.3 μM.

Several other flavone derivatives were examined (Table I). Flavone with no substituent (F-0) was much less inhibitory. Among the compounds tested cirsiliol (F-26) and pedalitin (F-27) were most active. These compounds have a catechol structure in the B ring which seems to be necessary to inhibit 5-lipoxygenase since compounds F-77 (R_5=hydrogen, R_6=hydroxyl), F-79 (R_5= hydroxyl, R_6=methoxyl) and F-75 (R_5=R_6=methoxyl) were much less active. However, catechol itself was essentially inactive. It remains unclarified whether or not the flavone inhibitor functions as a polyphenolic antioxidant as described for soybean lipoxygenase (24). Furthermore, modification of the 5-hydroxyl group of cirsiliol decreased the inhibitory effect as in F-81 (R_1=methoxyl). Pedalitin (F-27) with 6-hydroxyl group was as active as cirsiliol. Demethylation at the 7-position reduced the inhibitory effect as noted in F-28.

Cirsiliol as a 5-lipoxygenase inhibitor was examined at the tissue level. Fragments of lung isolated from passively sensitized guinea pig were challenged by antigen, and the release of SRS-A was followed by contraction of guinea pig ileum. As shown in Fig. 3, when cirsiliol was added to the reaction mixture prior to the challenge with antigen (10 μg/ml), SRS-A release was reduced in a dose-dependent manner (IC_{50}, about 0.4 μM). A similar result was obtained with a smaller amount of antigen (1 μg/ml). There was no antagonistic action of cirsiliol at a concentration of 32 μM when the ileum was contracted by the addition of guinea pig SRS-A equivalent to 0.4 nM leukotriene D_4. Histamine release caused by the antigen challenge (10 μg/ml) was reduced by only 27% in the presence of 10 μM cirsiliol. The IC_{50} at the tissue level (about 0.4 μM) was a little higher than the value at the enzyme level (about 0.1 μM). The difference may be attributed to a

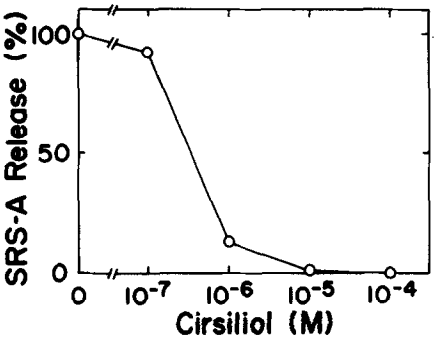


Fig. 3 Inhibition by cirsiliol of the immunological release of SRS-A from the lung of sensitized guinea pig. The lung fragments were preincubated with cirsiliol at concentrations as indicated, followed by the challenge with antigen (10 μg/ml). Production of SRS-A was examined by contraction of ileal strip of guinea pig, and each point represents the mean value of duplicate determinations. The ileal contraction observed without preincubation with cirsiliol was expressed as 100%.

disturbed permeability of the compound into the cell or to its intracellular metabolism to a less effective compound.

As compared with cirsiliol, earlier reported quercetin and baicalein were much less effective to inhibit the production of SRS-A; 92% inhibition with 100 μM quercetin (10) and 77% with 370 μM baicalein (9). Quercetin was not so active as cirsiliol (Table I). Baicalein was reported to inhibit platelet 12-lipoxygenase, and a possible effect of this compound on 5-lipoxygenase was mentioned but not demonstrated experimentally (25). Judging from the structure-activity relationship described above, baicalein without vicinal diol in the B ring of flavone is presumably a less potent inhibitor. It should be noted that flavone derivatives similar to those used in our work inhibited aldose reductase from rat and bovine lens (26). Therefore, we should be careful in the interpretation of experimental findings with a flavone compound as 5-lipoxygenase inhibitor although it may be a useful biochemical and pharmacological tool to elucidate the role of leukotriene in certain physiological and pathological events. The in vivo effect of the new 5-lipoxygenase inhibitor and organic chemical derivatization of the compound for more potent and specific inhibitors are now under investigations.

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