Biochemical Pharmacology, Vol. 34, No. 14, pp. 2477–2482, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

ACTIONS OF FLAVONOIDS AND THE NOVEL ANTI-INFLAMMATORY FLAVONE, HYPOLAETIN-8-GLUCOSIDE, ON PROSTAGLANDIN BIOSYNTHESIS AND INACTIVATION

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(*Received* 6 *November* 1984; *accepted* 7 *February* 1985)

Abstract—The newly identified plant-derived flavone-glycoside hypolaetin-8-glucoside, which has anti-inflammatory and gastric ulcer protective properties, and its corresponding aglycone, hypolaetin, were tested for effects on prostaglandin biosynthesis and degradation. They were compared with four other flavonoids, viz. rutin and its corresponding aglycone, quercetin, and the aglycones isoscutellarein and kaempferol. Over the range 10–1000 μM the glycosides rutin and hypolaetin-8-glucoside stimulated prostaglandin formation by sheep seminal vesicle microsomes incubated with radiolabelled arachidonic acid; the other compounds were essentially inactive. Over 5–5000 μM rutin and hypolaetin-8-glucoside enhanced the release of prostacyclin (and other prostanoids) from fragments of rat caecum incubated in the absence of additional arachidonic acid; the four aglycones compounds did not stimulate prostacyclin release but some reduced it at 5000 μM. However, the glycosides did not affect the enzymatic inactivation of radiolabelled prostaglandin $F_{2\alpha}$ by semi-purified bovine lung prostaglandin 15-hydroxydehydrogenase (PGDH) or in 100,000 *g* supernatants prepared from homogenised rat stomach. Three of the four aglycones (quercetin, kaempferol, isoscutellarein, in descending order of potency) were inhibitory to PGDH with ID_{50} values in the range 130–2100 μM. The results show that the capacity of flavonoids to enhance prostaglandin formation is associated with the presence of glycosidic substitution, whereas PGDH inhibition requires its absence. The relevance of this biochemical profile of hypolaetin-8-glucoside to its anti-inflammatory gastroprotective effects *in vivo* is discussed.

Previous studies by Villar and co-workers have shown that extracts of the Spanish herb *Sideritis mugronensis* exert anti-inflammatory, analgesic and gastroprotective actions in the rat [1, 2], and that this may be ascribed to at least two constituents, diterpenoids [3] and flavonoids [4]. More recently, a novel flavonoid (hypolaetin-8-glucoside) was isolated from *S. mugronensis* [5] and was shown to possess these properties [6]. These findings may provide a scientific basis for the use of decoctions of this plant as an antirheumatic and digestive agent in Spanish folk medicine. The previous studies did not address the question of the mechanism of action of the plant extracts or of the hypolaetin-8-glucoside component. However, there is a considerable interest in the role of the cyclo-oxygenase and lipoxygenase products of arachidonic acid metabolism in both acute [7, 8] and chronic [9] inflammatory diseases and as cytoprotective and anti-secretory agents in the stomach [10, 11], and a number of recent studies have suggested that flavonoids may interact directly with the prostaglandin system. Thus, various flavonoids have been shown both to inhibit and to substitute as cofactors for prostaglandin generation [12, 13], to inhibit arachidonate lipoxygenation [14, 15] and to inhibit enzymes involved with inactivation or biotransformation of prostaglandins [16].

The present paper describes an investigation of the actions of hypolaetin-8-glucoside and its corresponding aglycone, hypolaetin, on prostaglandin biosynthesis and inactivation in a number of test systems. For comparative purposes, rutin (quercetin-3-rutinoside) and its aglycone, quercetin, as well as the aglycones isoscutellarein and kaempferol were also tested.

MATERIALS AND METHODS

Drugs. The [1-^{14}C]arachidonic acid (sp. act. 58 mCi/mmole) and [^{3}H-9β]-prostaglandin $F_{2\alpha}$ (sp. act. 14.8 Ci/mmole) were obtained from Amersham International (U.K.). Reduced glutathione, NAD^+, arachidonic acid, prostaglandin $F_{2\alpha}$, rutin and quercetin were from Sigma London Ltd. (Poole, Dorset, U.K.), and kaempferol was obtained from Sarsyntex (Bordeaux, France). Hypolaetin-8-glucoside, hypolaetin and isoscutellarein were purified from *S. mugronensis* in the Department of Pharmacognosy and Pharmacodynamics, University of Valencia, Spain.

Effects of drugs upon sheep seminal vesicle cyclo-oxygenase activity. Portions of frozen sheep seminal vesicle (1–4 g), obtained from Northeast Biomedical Labs. Ltd. (Uxbridge, U.K.), were allowed to thaw, homogenised in 4 vol. pH 7.4 50 mM Tris–HCl, and the 100,000 *g* pellet prepared as described [17]. Resuspended microsomes were incubated in 0.2 ml final volume (1.3 ± 0.3 mg microsomal protein per tube) with 10 μg/ml arachidonic acid containing

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0.02 μCi ^{14}C-arachidonic acid (0.1 μg), 3 mM reduced glutathione and appropriate amount of flavonoids for 60 min at 37°, after which conversion to prostaglandins was assayed by radio-t.l.c. after extraction of the reaction mixture [18]. A total of 13 sets of experiments were performed and the extent of conversion of arachidonic acid to prostaglandin-like material (mainly PGE_2 on account of the presence of GSH) ranged from 20.3 to 85.0% with a mean value of 59.0 ± 5.7%. In the Results section, the effects of drugs on prostaglandin biosynthesis have been normalised and are expressed in terms of per cent of the control value obtained in each reaction set; each drug was tested in quadruplicate at each concentration and on several different microsomal preparations.

Effect of drugs upon release of prostaglandins by fragments of rat caecum. Fragments of rat caecum weighing 15–70 mg (mean 33.7 ± 0.8, N = 274) were cut from thawed rat caecum that had been cleaned and stored frozen, and were placed in 0.4 ml vol. of appropriate concentrations of drugs dissolved in pH 7.5 50 mM Tris–HCl buffer. After preincubation for 60 min at 4°, the fragments were transferred to fresh tubes containing the same drugs and incubated for 15 min at 37° with occasional shaking. The caecum fragments were then withdrawn, blotted and weighed, and the solutions kept at −20° for subsequent radioimmunoassay for 6-keto $PGF_{1\alpha}$, the stable hydrolysis product of prostacyclin. This was achieved using 1–3 μl aliquots of the solutions taken in triplicate without extraction (the presence of drugs did not interfere with the assay), adding them to 100 μl pH 6.8 50 mM Tris diluent containing 1.0 g/l gelatine and 0.1 g/l sodium azide, and subjecting the samples to a double antibody procedure as described [19]. In the present experiments the inter-assay coefficient of variation for 6-keto-$PGF_{1\alpha}$ radioimmunoassay was 14.4%, the intra-assay coefficient of variation for triplicate samples was 14.9% and the cross-reactivity with the other measured prostaglandins was: thromboxane B_2 1.4%, PGE_2 4.0%, $PGF_{2\alpha}$ 1.1%. Five sets of experiments were performed using caeca obtained from 24 animals; in control tubes the amount of prostacyclin formed ranged from 1.14 to 2.94 ng/mg tissue/15 min (as immunoassayable 6-keto $PGF_{1\alpha}$) with a mean value of 2.1 ± 0.4 ng/mg/15 min. Radioimmunoassays for thromboxane B_2, prostaglandin E_2 and prostaglandin $F_{2\alpha}$ were also performed on certain selected samples. Results are normalised and expressed as percent of control as indicated in the section above describing experiments on cyclo-oxygenase activity.

Effects of drugs upon prostaglandin $F_{2\alpha}$ inactivation. Semi-purified bovine lung prostaglandin 15-hydroxydehydrogenase (PGDH) was obtained from B.D.H. (Dagenham, U.K.), and had an activity of 75 mU/ml (1.9 mU/mg). Rat stomach 100,000 *g* supernatants were prepared in pH 7.5 50 mM phosphate buffer containing 1 mM cysteine and 1 mM EDTA as described previously [17]. These preparations were incubated for 75 min at 37° with 10 μg/ml prostaglandin $F_{2\alpha}$ containing 0.1 μCi ^{3}H-prostaglandin $F_{2\alpha}$ (2.4 ng) and 5 mM NAD^+ together with appropriate amounts of drugs in a volume of 0.2 ml. After extraction, the extent of prostaglandin $F_{2\alpha}$ inactivation was measured by radio-t.l.c. [17]. As before, results are normalised and expressed as per cent of control as indicated above for experiments on cyclo-oxygenase activity. Seven sets of experiments were performed using bovine lung PGDH and inactivation of prostaglandin $F_{2\alpha}$ ranged from 23.0 to 49.3% (mean 35.2 ± 3.7%); six sets of experiments were performed using rat stomach 100,000 *g* supernatants and inactivation of prostaglandin $F_{2\alpha}$ ranged from 52.4 to 76.2% (mean 62.6 ± 3.7%).

Statistical treatment of data. Differences between drug-treated and control values were calculated from the mean of each quadruplicate control and test drug value within each reaction set, and the overall differences for the whole group were then obtained and subjected to Student's paired *t*-test, taking $P < 0.05$ as significant.

RESULTS

The structures of the six flavonoids used in this study are given in Fig. 1 which is arranged to distinguish the flavonols (-OH at position 3) from the flavones (no substituent at position 3), and the glycosides from the aglycones. Similarly, the results shown in Tables 1 and 2 are arranged to distinguish the flavonols from the flavones.

The two glycoside flavonoids, rutin and hypolaetin-8-glucoside, both stimulated prostaglandin formation from exogenous arachidonic acid in the sheep seminal vesicle microsomal system (Table 1). Stimulation of cyclo-oxygenase was progressive as a function of dose, and greater at the largest dose tested (1000 μM). The aglycone flavonoids had little effect, except that at 1000 μM both quercetin and kaempferol slightly but significantly inhibited prostaglandin biosynthesis.

This pattern of results was confirmed using a system in which the tissue is essentially intact and no exogenous arachidonic acid is provided. It has been shown previously that the generation of prostacyclin by fragments of rat caecum incubated in Tris buffer at pH 7.5 and 37° is dependent upon the activity within the tissue of cyclo-oxygenase which can be inhibited by aspirin-like drugs or enhanced by phenolic cofactors [20]. In the present experiments both rutin and hypolaetin-8-glucoside augmented the generation of prostacyclin (measured by RIA as 6-keto-$PGF_{1\alpha}$), but the dose–effect relationships were different (Table 1). Rutin produced a monophasic pattern of stimulation, reaching a × 3.7-fold increase at 5000 μM, whereas the effect of hypolaetin-8-glucoside peaked at 500 μM (increase over non-drug treated levels of × 2.3) but was less at 5000 μM. No stimulation of prostacyclin generation occurred in the presence of 5–5000 μM of the other four aglycone flavonoids, although quercetin and kaempferol both significantly inhibited prostacyclin production at 5000 μM. Similar conclusions were drawn when selected incubation sets were subjected to radioimmunoassay for other less abundant cyclo-oxygenase products, i.e. thromboxane B_2, prostaglandin E_2 and prostaglandin $F_{2\alpha}$. Control rates of release of these substances were 0.18 ± 0.03, 0.24 ± 0.02 and 0.28 ± 0.03 ng/mg/15 min, respectively (6-keto-$PGF_{1\alpha}$ control rate was 2.1 ± 0.4 ng/mg/15 min).

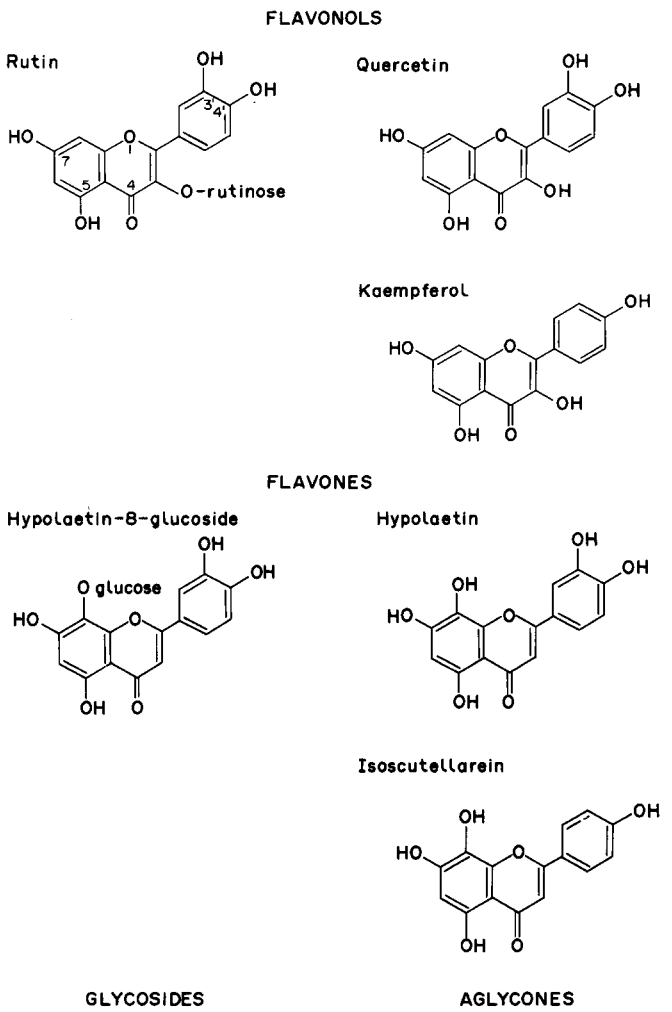

Fig. 1. Structures of the six flavonoids used in these experiments.

Output of all three prostaglandins was considerably augmented by rutin (at 5000 μM increases of × 2.3, × 12.1 and × 21.4, respectively) and by hypolaetin-8-glucoside at 500 μM (increases of × 2.4, × 4.7 and × 3.1, respectively). However, at 5000 μM hypolaetin-8-glucoside, the output of prostaglandin E_2 was not as large, although the elevated levels of thromboxane B_2 and prostaglandin $F_{2\alpha}$ persisted (cf. 6-keto-$PGF_{1\alpha}$, Table 1).

The six compounds were also tested in two PGDH systems, i.e. semi-purified bovine lung PGDH and the 100,000 g cytosolic supernatant prepared from homogenised rat stomach. The results in the two systems were essentially similar. The glycosides rutin and hypolaetin-8-glucoside were inactive and did not demonstrate any dose-related effects. The same was true for the aglycone hypolaetin. In contrast, quercetin and kaempferol produced dose-related inhibition of PGDH in both test systems, whereas isoscutellarein was active only in the rat stomach preparation. The results for the three aglycones in the rat stomach cytosolic preparation are given in Table 2, and yield approximate ID_{50} values of 130 μM for quercetin, 200 μM for kaempferol and 2100 μM for isoscutellarein.

DISCUSSION

The present results demonstrate that flavonoid compounds have varied actions on prostaglandin biosynthesis and degradation, and show that qualitatively different properties occur in glycoside vs aglycone flavonoids. The data also show that the novel anti-inflammatory anti-ulcer flavonoid, hypolaetin-8-glucoside, has characteristic stimulatory effects on prostaglandin synthesis but is inactive against PGDH.

Our data on quercetin and rutin in Table 1 complement those of Baumann *et al.* [12] who studied the ability of various phenolic substances to act as "cofactors" or "activators" in an "incomplete" prostaglandin E generating system in rat renal medulla homogenates incubated with labelled arachidonic acid in the presence of reduced glutathione (i.e. similar conditions to those used in the present experiments). They showed that rutin acted as an

Table 1. Effect of flavonoids on prostaglandin biosynthesis from sheep seminal vesicle microsomes (SSV) and release of prostacyclin from fragments of rat caecum

	SSV microsomes		Rat caecum fragments	
	μM	% of control	μM	% of control
Flavonols				
	10	95 ± 11	5	114 ± 31
Rutin	100	143 ± 22*	50	158 ± 37
(glycoside)	1000	182 ± 27**	500	273 ± 62*
			5000	375 ± 49*
	10	88 ± 12	5	86 ± 26
Quercetin	100	105 ± 15	50	104 ± 39
(aglycone)	1000	73 ± 15*	500	73 ± 19
			5000	26 ± 14*
	10	111 ± 11	5	74 ± 19
Kaempferol	100	110 ± 13	50	110 ± 11
(aglycone)	1000	75 ± 7*	500	98 ± 20
			5000	41 ± 15*
Flavones				
Hyplaetin-	10	87 ± 10	5	118 ± 18
8-glucoside	100	131 ± 17*	50	162 ± 32
(glycoside)	1000	156 ± 22**	500	235 ± 31*
			5000	125 ± 21
	10	101 ± 5	5	110 ± 12
Hypolaetin	100	106 ± 8	50	115 ± 32
(aglycone)	1000	117 ± 15*	500	129 ± 19
			5000	71 ± 9*
	10	109 ± 9	5	101 ± 6
Isoscutellarein	100	115 ± 10	50	80 ± 4
(aglycone)	1000	119 ± 11*	500	131 ± 9
			5000	not tested

Results show mean ± S.E.M. for 12–28 determinations on three to seven reaction sets (SSV) or four to 16 determinations (rat caecum) and are expressed as per cent of control. *$P < 0.05$, **$P < 0.01$.

effective cofactor, with similar activity to adrenaline or *p*-hydroxybenzoic acid, but that quercetin was inhibitory. However, in that study [12] the flavonoids were only tested at a single concentration (1 mM) so it was not possible to determine relative potencies or to check that the concentration–effect curves were monophasic. Similar effects of rutin (stimulatory) and quercetin (inhibitory) were also noted for prostaglandin biosynthesis in delipidated bovine seminal vesicle microsomes [16].

Further comparisons with the work of Baumann *et al.* are possible. Our results showing cyclo-oxygenase stimulation by the two glycosides rutin and hypolaetin-8-glucoside are in accordance with their suggestion [12, 13] that this property depends upon the presence in a phenol of a polar side-chain in either meta- or para- positions. This condition is satisfied by the two glycosides but not by the other four flavonoids that we tested. In our experiments on sheep seminal vesicle cyclo-oxygenase, quercetin and kaempferol were both inhibitory at 1000 μM (Table 1), as shown for the same compounds in the rat renal medulla assay [12, 13]. These two flavonoids possess the *ortho*-hydroxyl group (pyrocatechin), postulated as necessary for cyclo-oxygenase inhibition in the flavonoid series [21].

We obtained additional evidence for the ability of the two glycosides to enhance prostaglandin gen-

Table 2. Inhibition of rat stomach cytosolic PGDH activity by aglycone flavonoids

Concentration (μM)		$PGF_{2\alpha}$ inactivation (per cent of control)	Concentration (μM)		$PGF_{2\alpha}$ inactivation (per cent of control)
Quercetin	25	76.2 ± 6.0	Hypolaetin	10	73.2 ± 9.0
Quercetin	250	40.8 ± 7.0*	Hypolaetin	100	79.5 ± 4.0*
Quercetin	2500	8.6 ± 5.1**	Hypolaetin	1000	89.5 ± 4.5
Kaempferol	10	76.5 ± 7.0	Isoscutellarein	10	89.0 ± 10.0
Kaempferol	100	58.0 ± 3.0*	Isoscutellarein	100	71.0 ± 4.0**
Kaempferol	1000	38.7 ± 5.0**	Isoscutellarein	1000	57.3 ± 5.6*

Results show mean ± S.E.M., four determinations from experiments performed on one to four occasions. *$P < 0.05$, **$P < 0.01$ with respect to control tubes lacking flavonoids. Flavonols on left side of Table, flavones shown on right side (see Fig. 1 for structures).

eration by using the rat caecum fragment assay. This simple technique has the advantage that the tissue is essentially intact (although clearly traumatised) and that there is no requirement for cofactors or exogenous substrate. This latter point is important, since in a number of systems exogenous arachidonic acid appears to be handled in a different manner to endogenously released substrate (perhaps by a different pool of cyclo-oxygenase enzyme), and there is also evidence that alterations of exogenous substrate concentrations can cause marked differences in the product profile and in the sensitivity to drugs. In essence, our results with the caecum assay (Table 1) confirmed the stimulatory effect of the glycosides and showed also that at the highest dose tested (5000 μM), quercetin, hypolaetin and kaempferol were inhibitory. Furthermore, we have also found that the flavone glycoside naringin enhances the output of prostacyclin and other prostanoids from rat caecum *in vitro*, whereas the aglycone naringenin was inactive*.

Again, the simplest explanation for this data is that the stimulatory glycosides enhance prostacyclin formation by acting as cofactors. An alternative explanation, namely that the compounds favour redirection away from other prostanoids towards prostacyclin, can be discounted because we showed that the three other principal prostanoids (prostaglandins E_2 and $F_{2\alpha}$ and thromboxane B_2) are quantitatively minor in this tissue and that their formation was also enhanced in the presence of the glycosides. Other phenolic substances have been shown to enhance caecum prostacyclin formation by mechanisms thought to depend upon their ability to scavenge free radicals [20, 22], thus protecting cyclooxygenase from self-inactivation [23]. Since flavonoids are known to act as free radical scavengers [13, 24], it will be relevant to assess the compounds used in this study as scavengers of peroxy systems relevant to arachidonate metabolism.

Selective effects of the flavonoids on enzymes involved in prostaglandin inactivation were also observed, in that the two glycosides were inactive whereas among the aglycones, the flavonols were more active inhibitors than the flavones. Previous work has established that rutin and quercetin inhibit rabbit kidney prostaglandin 9-hydroxydehydrogenase but are not active against rat renal prostaglandin 9-hydroxydehydrogenase or rabbit colon prostaglandin 15-hydroxydehydrogenase [16].

Our results may also be relevant to the beneficial actions of hypolaetin-8-glucoside *in vivo*. It was previously shown in the rat that this novel flavonoid exerts an acute anti-inflammatory effect comparable to phenylbutazone in that at similar doses it suppressed the early phase of adjuvant-carrageenan-induced inflammation (although it had less effect on the prolonged phase) and reduced carrageenan-induced abscesses [6]. However, unlike phenylbutazone, there was no evidence of gastric damage. In fact, hypolaetin-8-glucoside proved to protect against cold stress-induced ulcers in the same species, with a potency only 2.4 times less than cimetidine. It seems reasonable to suggest that the capacity of hypolaetin-8-glucoside to enhance prostaglandin production could be a basis for the gastroprotective effects since prostaglandins, notably prostacyclin and prostaglandin E_2, are formed in gastric tissue and have anti-secretory, anti-ulcer and cytoprotective properties [10, 11].

This type of explanation does not apply to the anti-inflammatory effects of hypolaetin-8-glucoside because in acute inflammatory episodes prostaglandins enhance the signs and symptoms [7, 8], even though they may have anti-inflammatory effects in chronic inflammation [9, 25] and may also modulate humoral immune mechanisms [26]. Thus hypolaetin-8-glucoside may owe its anti-inflammatory effect to a different mechanism, independent of interactions with prostaglandins. It will therefore be important to assess the effectiveness of interactions of hypolaetin-8-glucoside with other pro-inflammatory biochemical pathways so that the mode of action of this new type of anti-inflammatory drug can be clarified.

Acknowledgements—M. J. Alcaraz thanks the Spanish Government (Ministerio de Educacion y Ciencia) for a fellowship, and Prof. A. Villar and Miss M. A. Gasco (Department of Pharmacognosy and Pharmacodynamics, University of Valencia, Spain) for their collaboration in obtaining the natural products used.

* J. R. S. Hoult, unpublished experiments.

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