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Some aspects of the inhibitory activity of hypolaetin-8-glucoside in acute inflammation

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Hypolaetin-8-glucoside (H-8-G) has been examined for its mode of action in several models of acute inflammation. Its anti-inflammatory activity in carrageenan-induced inflammation of the rat hind-paw is not affected either by adrenalectomy or by phentolamine given with propranolol. H-8-G and its aglycone, hypolaetin, did not antagonize the actions of histamine, 5-hydroxytryptamine (5-HT), bradykinin or prostaglandin E_2 (PGE_2) on various smooth muscle preparations in-vitro, but protected erythrocytes from heat-induced lysis. The glycoside was more potent than troxerutin on capillary permeability increased by histamine and exerted inhibitory effects on protein exudation, leucocyte migration and β-glucuronidase activity in the carrageenan air pouch, thereby showing some difference from indomethacin. These results are discussed in relation to the features of non-steroidal anti-inflammatory drugs (NSAID) and flavonoid anti-inflammatory actions.

The general feature of non-steroidal anti-inflammatory drugs (NSAID) is their ability to inhibit the cyclo-oxygenase pathway (Vane 1971) which is thought to parallel their anti-inflammatory potencies (Barbieri et al 1977). On the other hand it has been postulated that inhibition of prostaglandin synthesis is linked with the gastrointestinal side effects of such drugs (Whittle et al 1980).

Plants might provide alternative agents for the treatment of inflammatory disorders, as suggested by their use in folk medicine. Previously, we reported the isolation from *Sideritis mugronensis* Borja (*Lamiaceae*) of hypolaetin-8-*O*-glucoside (H-8-G; 5,7,3′,4′-tetrahydroxyflavone-8-*O*-β-D-glucoside), a compound possessing anti-inflammatory activity in rats (Villar et al 1984, 1985). In contrast to NSAID, this flavonoid lacks ulcerogenic effects and protects against cold-restraint-induced gastric ulcers (Villar et al 1984).

H-8-G appears to have a novel mode of action compared with aspirin-like drugs in that the glycoside and its aglycone, hypolaetin, do not inhibit cyclo-oxygenase activity (Alcaraz & Hoult 1985).

The present experiments were designed to allow more insight into the mode of action of H-8-G underlying its anti-inflammatory properties.

MATERIALS AND METHODS

Hind-paw oedema induced by carrageenan in adrenalectomized rats

Male Wistar rats, 200–250 g, were bilaterally adrenalectomized or sham-operated under anaesthesia and used 7 days after the operation. Hind-paw oedema was induced by injection of 0·1 mL 2% carrageenan (w/v in 0·9% NaCl (saline)) according to Winter et al (1962). H-8-G (60 mg kg^{-1} i.p.) was given to groups of 6 animals 1 h before carrageenan injection and paw volume was recorded plethysmographically 1 and 3 h later.

Hind-paw oedema induced by carrageenan in rats given phentolamine and propranolol

Groups of 6 female Wistar rats, 160–190 g, were used. 1 h before the induction of the hind-paw oedema by carrageenan (Winter et al 1962), rats were injected with 0·1 mL of the α-blocking drug phentolamine (10 mg kg^{-1} s.c.) and 0·1 mL of the β-blocking drug propranolol (10 mg kg^{-1} s.c.). Either H-8-G or vehicle was given intraperitoneally at the same time.

Capillary permeability increased by histamine

Capillary permeability was increased according to Tarayre & Lauressergues (1977) by administration of histamine to female Wistar rats, 200–220 g, distributed in groups of 6 animals. After i.v. administration of 0·1 mL of Evans blue (60 mg kg^{-1}) suspended in saline, 6 wheals were produced by intradermal injection of 0·1 mL of histamine (500 μg mL^{-1}) per wheal into the shaved abdominal skin. 20 min later animals were killed and the extravasated dye of the punched out skin was estimated. To each piece of skin (1·5 cm) containing the lesion, 4 mL of formamide was added and the tubes were

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incubated at 65 °C for 24 h. The optical density was measured at 620 nm. H-8-G (100, 200 and 300 mg kg^{-1}) and troxerutin (200, 300 and 400 mg kg^{-1}) were administered subcutaneously 1 h before administration of Evans blue.

Carrageenan pouch

Female Wistar rats, 170–200 g, were used in groups of 6–9 following the modified method of Fukuhara & Tsurufuji (1969). 4 mL of 2% (w/v) carrageenan in saline were injected into an air sac (8 mL volume) previously formed in the back of the rats by injecting air subcutaneously 24 h before. Immediately after injection of the irritant, H-8-G (50, 100 and 150 mg kg^{-1}) and indomethacin (2, 4 and 6 mg kg^{-1}) were administered intraperitoneally. After 6 h, rats were killed and the inflammatory exudate collected. This was diluted twice with saline and centrifuged at 2000 rev min^{-1} for 20 min at 4 °C.

Leucocyte counts. Supernatants were removed and 20 μL aliquots taken from each tube, diluted (1/500, v/v) with isotonic solution and total leucocyte numbers estimated using Coulter automatic counting.

Protein content. Aliquots, 10 μL, of supernatants were assayed for protein using the method described by Lowry et al (1951), with bovine serum albumin as the reference standard.

β-*Glucuronidase activity.* Aliquots, 50 μL, of supernatants were assayed for this enzyme activity following the method of Barrett & Heath (1979). The substrate was 4-methylumbelliferyl-β-D-glucuronide. 50 μL volumes of a stock solution (5 mM) in citrate buffer, pH 5·0, were incubated with 50 μL of sample supernatants for 25 min at 37 °C. Reaction was stopped by adding 1 mL of a bicarbonate solution (0·5 M $NaHCO_3$ + 0·5 M Na_2CO_3, 1:1 volumes). Readings were then taken using a spectrofluorimeter (excitation 356 nm, emission 500 nm).

Smooth muscle assays

The influence of H-8-G and its aglycone, hypolaetin, was determined on cumulative dose-response curves (van Rossum 1963) to histamine (in guinea-pig ileum and rat uterus) and PGE_2 and 5-HT (in rat uterus). We also studied the effect of these flavonoids on single dose–response curves of bradykinin in the rat isolated uterus using the conventional method (Turner 1965). Concentrations assayed were $2·5 \times 10^{-7}$–$2·5 \times 10^{-5}$ M.

Hyperthermic haemolysis of normal erythrocytes

We used the method described by Ito et al (1982). Blood was freshly obtained from male Wistar rats (200–220 g) by retro-orbital puncture with a heparinized pipette and the erythrocytes were separated by centrifuging (1500 rev min^{-1}, 15 min). They were washed twice with saline and a 5% suspension was made up in phosphate buffer (0·15 M, pH 7·4). The drugs were dissolved in buffer by the addition of dimethylsulphoxide to give a final concentration of 5%. 2·7 mL of erythrocyte suspension was mixed with 0·3 mL of drug solution. The mixture was shaken gently and kept at room temperature (20 °C) for 15 min. It was then heated at 50–51 °C for 20 min, cooled in ice water and centrifuged at 3000 rev min^{-1} for 20 min. The optical density of the supernatant was measured at 540 nm. Experiments were in triplicate and for comparative purposes, hypolaetin, indomethacin, phenylbutazone and butibufen were also included.

Heat denaturation of serum albumin

According to Nagatomi & Ando (1984), test compounds were dissolved in distilled water and a small quantity of dimethylsulphoxide was added up to 10% to dissolve them completely. To 1 mL of bovine serum albumin in 0·2 M phosphate buffer (pH 5·3) was added 1 mL of drug solution. After being kept at room temperature (20 °C) for 15 min, the mixture was heated at 62–63 °C in a waterbath for 6 min and after cooling the turbidities of the samples measured at 660 nm. Experiments were in triplicate.

Inhibition of the proteolytic enzyme trypsin

To test for possible inhibition of trypsin by the drugs listed and H-8-G, the modified method of Brown & Pollock (1970) with haemoglobin as substrate was used. To 1·8 mL of trypsin (1 mg mL^{-1}) in 0·015 M phosphate buffer (pH 8·0) were added test compounds dissolved in 0·2 mL of the same buffer and a small quantity of dimethylsulphoxide up to 10%. The mixture was preincubated at 37 °C for 10 min and then 1 mL of haemoglobin (30 mg mL^{-1}) was added and the mixture incubated for 20 min at 37 °C. The enzyme reaction was terminated by the addition of 2 mL of 5% trichloroacetic acid and after being kept for 30 min at room temperature, tubes were centrifuged at 3000 rev min^{-1} for 10 min to obtain the clear supernatant. To 0·5 mL of supernatant were added 2·5 mL of 0·55 M Na_2CO_3 solution and 1 mL of Folin reagent to read the optical density at 660 nm after 30 min.

Drugs

The drugs used were: troxerutin (Mack, Pfizer, S.A.); butibufen (Juste), bradykinin triacetate (Aldrich Chem.), 5-hydroxytryptamine creatinine sulphate (Vister), histamine dihydrochloride (Merck); phenylbutazone and phentolamine methanesulphonate (Ciba-Geigy); indomethacin, (±)-propranolol hydrochloride, prostaglandin E_2, 4-methylumbelliferyl-β-D-glucuronide, Evans blue, bovine haemoglobin, bovine serum albumin and trypsin (Sigma Chem.); Folin-Ciocalteau reagent (Pan reac); and carrageenan (Marine Colloids). H-8-G and hypolaetin were purified from *Sideritis mugronensis* (Villar et al 1985).

Statistical analysis of data

The results were statistically evaluated using the Student's *t*-test for unpaired samples.

RESULTS

Hind-paw oedema induced by carrageenan in adrenalectomized rats

The rate of swelling of paws produced by carrageenan in adrenalectomized rats was similar to that in sham-operated animals. In adrenalectomized rats the inhibitory activity of H-8-G (60 mg kg^{-1} i.p.) did not disappear (Table 1).

Hind-paw oedema induced by carrageenan in rats given phentolamine and propranolol

Phentolamine and propranolol (10 mg kg^{-1} s.c.) given together at the same time as H-8-G (60 mg kg^{-1} i.p.), had no influence on the reduction of swelling mediated by H-8-G in carrageenan-induced paw oedema in rats (Table 1).

Capillary permeability increased by histamine

As shown in Table 2, H-8-G was more active than troxerutin, a clinically used flavonoid derivative which reduces capillary fragility and permeability. Both compounds exerted dose-related effects.

Table 1. Effect of H-8-G on carageenan-induced hind-paw oedema in sham-operated, adrenalectomized and phentolamine + propranolol-treated rats (n = 6).

	% Inhibition (mean ± s.e.m.) at:	
Control	1 h	3 h
(H-8-G alone)	21·14 ± 7·05	33·84 ± 8·07**
H-8-G + phentolamine and propranolol	18·51 ± 2·24*	32·05 ± 4·17**
Adrenalectomized	35·26 ± 5·88**	48·99 ± 4·21**
Sham-operated	31·43 ± 5·35*	36·98 ± 3·26**

*$P < 0.05$, **$P < 0.01$.

Table 2. Effect of H-8-G and troxerutin on capillary permeability increased by histamine (n = 6).

Dose (mg kg^{-1}, s.c.)	% Inhibition (mean ± s.e.m.) H-8-G	Troxerutin
100	34·62 ± 12·92*	
200	42·85 ± 5·51**	22·50 ± 12·61
300	64·26 ± 2·45**	32·67 ± 7·23*
400		35·55 ± 5·42*

*$P < 0.05$, **$P < 0.01$.

Carrageenan pouch

Leucocyte counts. Table 3 shows the dose-dependent inhibitory effects of H-8-G and indomethacin on leucocyte migration in-vivo; these were significant at all doses assayed.

Protein content. Indomethacin significantly inhibited protein exudation into the carrageenan pouch at doses between 2–6 mg kg^{-1}, but for H-8-G the inhibition was significant only at the highest dose (150 mg kg^{-1}) (see Table 3).

β-Glucuronidase activity. H-8-G (100 and 150 mg kg^{-1} i.p.) and indomethacin (4 and 6 mg kg^{-1} i.p.) significantly inhibited the increase of β-glucuronidase activity in the carrageenan pouch fluid. At lower doses there was no significant effect (Table 3).

Smooth muscle assays

Neither H-8-G nor hypolaetin exerted any antagonism against several mediators of the inflammatory reaction (histamine, 5-HT, PGE_2, bradykinin) in the rat isolated uterus. This absence of effect was noted also after histamine had been applied to the guinea-pig isolated ileum (data not shown).

Hyperthermic haemolysis of normal erythrocytes

Hypolaetin, H-8-G, indomethacin, phenylbutazone and butibufen inhibited the haemolysis of normal erythrocytes at the concentrations 5×10^{-5}–5×10^{-4} M (Table 4). At lower concentrations, only H-8-G and indomethacin behaved as inhibitors in this test. The order of potency was: indomethacin > H-8-G > butibufen > hypolaetin > phenylbutazone.

Heat denaturation of serum albumin

Hypolaetin, phenylbutazone and butibufen significantly inhibited heat-induced denaturation of bovine

Table 3. Effect of H-8-G and *indomethacin* on leucocyte migration, protein content and β-glucuronidase activity of the pouch fluid by the carrageenan pouch method (n = 6–9).

Dose (mg kg^{-1}, i.p.)	% Inhibition (mean ± s.e.m.)					
	Leucocyte counts		Protein content		β-Glucuronidase activity	
	Indomethacin	H-8-G	Indomethacin	H-8-G	Indomethacin	H-8-G
2	55·74 ± 3·63**		27·14 ± 7·69*		19·32 ± 12·43	
4	67·82 ± 5·32**		48·55 ± 4·17**		29·62 ± 5·70*	
6	79·86 ± 7·24**		53·86 ± 4·44**		44·72 ± 7·81**	
50		48·12 ± 4·13*		7·97 ± 4·53		19·70 ± 6·00
100		63·92 ± 3·32**		16·36 ± 5·51		27·38 ± 4·37*
150		84·90 ± 2·52**		21·52 ± 6·56*		44·92 ± 4·58**

*$P < 0.05$, **$P < 0.01$.

Table 4. Effect of H-8-G, hypolaetin, phenylbutazone, butibufen and indomethacin on hyperthermic haemolysis of normal erythrocytes and heat denaturation of serum albumin (n = 6).

Drug	% Inhibition (mean ± s.e.m.)					
	Erythrocyte lysis			Albumin denaturation		
	5×10^{-4} M	5×10^{-5} M	5×10^{-6} M	$2{\cdot}5 \times 10^{-4}$ M	10^{-4} M	$2{\cdot}5 \times 10^{-5}$ M
H-8-6G	68·84 ± 1·56**	39·01 ± 0·98**	24·36 ± 0·83**	−38·96 ± 3·18*	−38·48 ± 1·43*	−44·79 ± 2·62*
Hypolaetin	22·61 ± 2·81**	9·66 ± 1·33**	2·61 ± 0·83	81·04 ± 0·51**	55·89 ± 2·10**	25·00 ± 1·56
Phenylbutazone	17·74 ± 2·05**	8·67 ± 1·80**	4·53 ± 2·49	92·76 ± 1·19**	55·47 ± 2·19**	37·71 ± 0·89*
Butibufen	43·34 ± 2·17**	17·21 ± 1·61**	7·04 ± 1·05*	74·58 ± 2·09**	64·95 ± 0·38**	47·13 ± 2·22**
Indomethacin	88·23 ± 0·65**	70·62 ± 2·98**	31·53 ± 1·41**	−90·98 ± 2·89**	−71·93 ± 1·14**	−66·13 ± 1·16**

*$P < 0.05$, **$P < 0.01$.

serum albumin at the concentrations $2{\cdot}5 \times 10^{-5}$–$2{\cdot}5 \times 10^{-4}$ M, but H-8-G and indomethacin increased the denaturation (Table 4).

Inhibition of the proteolytic enzyme trypsin

None of the drugs tested (indomethacin, H-8-G, hypolaetin, phenylbutazone and butibufen) was able to inhibit trypsin activity (data not shown).

DISCUSSION

To determine if the anti-inflammatory action of H-8-G was mediated by glucocorticoids secreted as a result of stimulation of the hypophysis-adrenal system, we compared the inhibitory effects of this flavonoid on carrageenan-induced hind-paw oedema in sham-operated and in adrenalectomized rats, and found that the anti-inflammatory actions of H-8-G were essentially the same in both groups, leading us to conclude that inhibition was not elicited via the adrenal glands.

Catecholamines can reduce carrageenan oedema in mice (Green 1972) and it has been reported that flavonoids possessing a catechol structure can inhibit catechol-*O*-methyltransferase (Gugler & Dengler 1973; Borchardt & Huber 1975) and thus potentiate the actions of noradrenaline. In the present experiments, however, the inhibitory activity of H-8-G on carrageenan-induced oedema was not affected by either phentolamine or propranolol. Consequently, it may be deduced that the anti-inflammatory action of this glycoside is not mediated by catecholamines or by turnover of arachidonate.

Histamine acts as an inflammatory mediator at the initial phase of the carrageenan rat paw oedema (Di Rosa et al 1971). It has a dominant role in eliciting vasodilatation (through interaction with both H_1- and H_2-receptors) and a subordinate role in mediating increased vascular permeability (through H_1-receptors) (Woodward et al 1982). Other endogenous substances such as kinins and prostaglandins are also thought to contribute to the increased vascular permeability in this experimental model of inflammation (Di Rosa et al 1971). H-8-G inhibited the histamine-induced increase in vascular permeability and carrageenan-induced inflammation, but this glycoside and its aglycone failed to antagonize the actions of histamine, 5-HT, bradykinin and PGE_2 on isolated smooth muscle (rat uterus and guinea-pig ileum). Thus, it is unlikely that the effects of H-8-G on inflammation would be dependent on antagonism

of such mediators. Our experiments also showed the absence of effect on trypsin activity related to the release of kinins.

As NSAID are inactive towards the exudation of plasma and proteins caused by histamine at doses equivalent to their ED50 on carrageenan oedema, we compared H-8-G with another flavonoid in this test as this class of compounds does reduce this histamine response but the mode of action has not been established (Tarayre & Lauressergues 1977); it does not seem to be dependent on regional blood flow changes (Gerdin & Svensjö 1983). We found H-8-G to be more potent than troxerutin, which is used clinically in conditions with inflammatory oedema, but the contribution of antihistamine activity or potentiation of catecholamines to the anti-oedematous effect, as suggested for flavonoids by Tarayre & Lauressergues (1977), should be discounted.

Prostaglandins may mediate infiltration of plasma protein induced by carrageenan (Lo et al 1982). In this respect it is noteworthy that H-8-G, which is unable to inhibit prostaglandin synthesis (Alcaraz & Hoult 1985), was less active than indomethacin in affecting this response. NSAID may diminish leucocyte accumulation through suppression of protein infiltration and therefore of chemotactic activity at the inflammatory site (Lo et al 1981), while H-8-G exhibited an inhibitory effect on leucocyte migration but very little inhibition of protein infiltration. Inhibition of neutrophil accumulation is independent of effects on cyclo-oxygenase activity (Higgs 1980) and may be related to reduction of cellular activities such as proliferation, blockade of chemotaxis or the generation of chemotactic factors (Almeida et al 1980). It could also depend on lipoxygenase inhibition in the presence of high doses of NSAID (Higgs 1980).

Lysosomal enzyme secretion by neutrophils depends on the activity of various pathways and the generation of arachidonate metabolites via cyclo-oxygenase and lipoxygenase (Ward et al 1985). NSAID, however, act in different ways according to the stimulus used (Kaplan et al 1984). These drugs show membrane stabilizing activity in rat liver lysosomes (Smith et al 1976) and protect erythrocytes from heat-induced lysis, which is an example of a more general stabilizing action on membranes (Ito et al 1982). Flavonoids also stabilize lysosomes (van Caneghem 1972) and exert different effects on neutrophil functions (Tauber et al 1984).

The anti-inflammatory activity of H-8-G was supported by our in-vitro experiments on the protection of erythrocytes from heat treatment. In this test, as well as in the heat denaturation of serum albumin, H-8-G behaved like indomethacin whereas hypolaetin exerted effects similar to those of phenylbutazone.

In summary, our findings suggest that H-8-G acts through inhibition of leucocyte migration into the inflamed site, in addition to membrane stabilizing effects at high doses.

Flavonoids can interact with a variety of biochemical systems sustaining the inflammatory process, e.g. free radicals generation (Havsteen 1983) and synthesis of arachidonic acid metabolites (Yamamoto et al 1984). There are also reports of their interference with divalent cation-sensitive steps of the regulation of cellular enzyme activities (Fewtrell & Gomperts 1977).

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