Influence of Anti-Inflammatory Flavonoids on Degranulation and Arachidonic Acid Release in Rat Neutrophils

Maria Tordera, Maria Luisa Ferrándiz and Maria José Alcaraz
Departamento de Farmacología de la Universidad de Valencia, Facultad de Farmacia, Avda. Vicent Estelles s/n, 46100 Burjassot, Valencia, Spain

Z. Naturforsch. 49c, 235–240 (1994); received December 30, 1993

Flavonoids, Anti-Inflammatory Flavonoids, Arachidonic Acid, Lysosomal Enzymes, Rat Neutrophil

We assessed the effects of 24 flavonoid derivatives, reported as anti-inflammatory, on lysosomal enzyme secretion and arachidonic acid release in rat neutrophils. Amentoflavone, quercetin-7-O-glucoside, apigenin, fisetin, kaempferol, luteolin and quercetin were the most potent inhibitors of β-glucuronidase and lysozyme release. The first compound was also able to inhibit basal release. These flavonoids besides chrysin and to a reduced extent, naringenin, significantly inhibited arachidonic acid release from membranes. A correlation between degranulation and arachidonic acid release was found for this series of compounds. Structure-activity relationships and implications for the anti-inflammatory effects of these flavonoids were discussed.

Introduction

Besides a wide range of biological activities (Havsteen, 1983) some members of the flavonoid class display anti-inflammatory effects in animals (Alcaraz and Jiménez, 1988; Ferrándiz and Alcaraz, 1991). The mechanism of action is unclear but it may involve the inhibition of eicosanoid production by intact cells with different selectivity towards cyclooxygenase or lipoxygenase pathways depending on their structural features (Moroney et al., 1988). Other actions possibly account for the anti-inflammatory effects of flavonoids. In this respect, recent attention has focused upon the inhibitory effects of flavonoids on reactive oxygen species generation in neutrophils (Limasset et al., 1993).

Secretion is a crucial event induced by leukocyte activation and resulting in the release of degradative enzymes which play an important role in tissue damage during inflammation. There are several possible links between arachidonic acid release and secretion. Thus, products derived from PLA₂ activity could be required for the fusion of lysosomes and plasma membranes, or arachidonic acid released would induce degranulation and other neutrophil responses (Smolen and Weissmann, 1993).

The objective of this study was to obtain evidence as to whether a series of flavonoids endowed with anti-inflammatory properties, modify select leukocyte responses which contribute to the inflammatory process. Thus, we have investigated the capacity of a series of flavonoids to inhibit degranulation in rat neutrophils and we have included as reference some compounds, like quercetin, previously reported as inhibitors of leukocyte functions in other species (Bennett et al., 1981; Middleton Jr. et al., 1987; Showell et al., 1981; Blackburn et al., 1987; Berton et al., 1980). In order to understand better the modification of eicosanoid generation by flavonoids in relevant inflammatory cells, we have also assessed in the present study the influence of these flavonoids on the release of arachidonic acid from lysocyte membranes, a process mainly dependent on PLA₂ activity.

Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; FMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; PBS, phosphate buffer saline; PLA₂, phospholipase A₂; PLC, phospholipase C; PLD, phospholipase D.

Reprint requests to Prof. Maria José Alcaraz.
Verlag der Zeitschrift für Naturforschung, D-72072 Tübingen
0939–5075/94/0300–0235 $03.00/0
Materials and Methods

Drugs

Sideritoflavone was isolated from *Sideritis leucantha* and hypolaetin-8-O-glucoside from *Sideritis mugronensis*, following known procedures (Vil­lar et al., 1985; Jiménez et al., 1986). Oroxindin from *Oroxyllum indicum*; quercetagetin-7-O-glucoside from *Tagetes erecta*, gossypin and hibifolin from *Hibiscus vitifolius*, and tambuletin from *Zanthoxylum alatum* were a gift from Prof. A.G.R. Nair (Pondicherry University, India). Other flavonoids were commercially available: flavone, 3-hydroxyflavone and fisetin (Aldrich); troxerutin (Almirall); quercetin (Merck); apigenin, luteolin and amentoflavone (Roth); leucocyanidol (Rovi); chrysin, rutin, morin, naringenin, naringin, (+)-catechin, (−)-epicatechin and kaempferol (Sigma). [1 4 C]-arachidonic acid (100 μCi/ml) was purchased from DuPont. Ionophore A 23187, BSA, glycogen, cytochalasin B, FMLP and triton X-100 were purchased from Sigma. The rest of the chemicals were of analytical grade.

Isolation of leukocytes

Rat peritoneal leukocytes elicited by glycogen (10 ml, 1%) were prepared by centrifugation and hypotonic lysis of contaminating red cells (Moroney et al., 1988). The cell suspension contained 90% neutrophils and viability was greater than 95% assessed by Trypan blue exclusion.

Lysosomal enzyme secretion

Leukocytes were suspended at 6 × 10⁶ cells/ml in PBS and preincubated for 8 min at 37 °C with cytochalasin B (5 μg/ml) followed by addition of test compound or vehicle (10 μl DMSO). After 8 min, FMLP (10⁻⁶ M) was added and incubation proceeded for 8 min. The cell suspensions were then placed on ice and centrifuged at 3000 r.p.m. for 15 min at 4 °C. β-Glucuronidase and lysozyme levels in supernatants were determined by spectrophotometric procedures previously described (Gianetto and de Duve, 1955; Yuli et al., 1982) and results were expressed as percentage of release with respect to tubes treated with 10 μl 20% triton X-100.

Arachidonic acid release (Croft et al., 1987)

Cells were suspended in Hank’s solution at a concentration of 10⁷ ml and incubated with arachidonic acid (0.1 μCi/ml) for 60 min at 37 °C. Leukocytes were then washed in PBS three times and resuspended in Hank’s solution + 0.1% BSA. Test compounds were preincubated for 15 min at 37 °C before addition of ionophore A 23187 (25 μg/ml). After 10 min, tubes were placed on ice and centrifuged as above. Radioactivity was determined in supernatants by liquid scintillation counting.

Results

In control incubations, the extent of lysozyme secretion was more than twice that of β-glucuronidase either in the presence or in the absence of stimulus (data not shown). A number of flavonoids inhibited β-glucuronidase and lysozyme release, taken as markers of rat peritoneal leukocytes degranulation (Table I). Amentoflavone, quercetagetin-7-O-glucoside, apigenin, fisetin, kaempferol, luteolin and quercetin yielded more than 60% of inhibition on both enzymes secretion. Besides, oroxindin, chrysin and hibifolin affected selectively β-glucuronidase release. The regression analysis of the curves obtained for these active compounds, showing more than 60% of inhibition at 10⁻⁴ M, at a range of concentrations between 10⁻⁵ M and 10⁻⁴ M (or 10⁻⁶ M and 10⁻⁴ M for amentoflavone), allowed us the calculation of the inhibitory concentration 50% (IC₅₀). Amentoflavone showed the highest potency, especially for inhibition of β-glucuronidase release, with an IC₅₀ in the μM range (Fig. 1). At concentrations between 3 × 10⁻³ M and 10⁻⁴ M amentoflavone gave percentages of inhibition for β-glucuronidase release higher than 100%, indicating its ability to influence degranulation in basal conditions (in the absence of any stimulus). In this case, its IC₅₀ was 4.5 ± 0.1 × 10⁻⁵ M and 6.2 ± 0.4 × 10⁻⁴ M, for β-glucuronidase and lysozyme basal release, respectively. For most com-
Table I. Structure of the flavonoids tested. Effect on leukocyte degranulation: percentage of inhibition at $10^{-4}$ M (% I) and inhibitory concentration 50% (IC$_{50}$).

<table>
<thead>
<tr>
<th>flavonols</th>
<th>R$_3$</th>
<th>R$_5$</th>
<th>R$_5$</th>
<th>R$_7$</th>
<th>R$_7$</th>
<th>R$_4$</th>
<th>% I</th>
<th>IC$_{50}$ (x 10$^{-5}$ M)</th>
<th>% I</th>
<th>Lysozyme IC$_{50}$ (x 10$^{-5}$ M)</th>
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<td>3'-vones</td>
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<td></td>
<td>56.7 ± 2.9**</td>
<td>N.D.</td>
<td>32.6 ± 1.0**</td>
<td>N.D.</td>
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<td>5-rutin</td>
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<td>72.0 ± 4.8**</td>
<td>6.6 ± 0.9</td>
<td>51.7 ± 3.3**</td>
<td>N.D.</td>
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<td>6-rutin</td>
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<td></td>
<td>92.9 ± 4.0**</td>
<td>2.6 ± 0.3</td>
<td>71.8 ± 3.3**</td>
<td>6.5 ± 0.7</td>
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<td>7-rutin</td>
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<td></td>
<td>90.9 ± 3.0**</td>
<td>3.5 ± 1.2</td>
<td>72.0 ± 1.3**</td>
<td>4.6 ± 0.3</td>
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<td>8-rutin</td>
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<td>32.7 ± 2.1**</td>
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<td>11.0 ± 5.9</td>
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<td>12.2 ± 2.4</td>
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<td>7.4 ± 1.1</td>
<td>44.3 ± 2.0**</td>
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<td>3'-vone</td>
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<td>103.2 ± 2.1**</td>
<td>6.7 ± 0.7</td>
<td>-54.2 ± 6.5**</td>
<td>N.D.</td>
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<td>5-rutin</td>
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<td></td>
<td>100.0 ± 5.2**</td>
<td>2.3 ± 0.3</td>
<td>101.7 ± 14.2**</td>
<td>3.3 ± 0.2</td>
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<td>74.4 ± 3.7**</td>
<td>4.5 ± 1.2</td>
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<td>90.4 ± 0.9**</td>
<td>2.4 ± 0.1</td>
<td>88.5 ± 2.7**</td>
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<td>36.3 ± 7.0**</td>
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<td>0.7 ± 3.3</td>
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<td>35.3 ± 11.9*</td>
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<td>12.3 ± 11.2</td>
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<td>62.4 ± 4.0**</td>
<td>7.1 ± 1.0</td>
<td>9.3 ± 6.2</td>
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<td>xanthesin</td>
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<td>56.7 ± 2.9**</td>
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<td>xanthesin</td>
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<td>10.2 ± 10.9</td>
<td>N.D.</td>
<td>20.5 ± 2.3</td>
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<td>14.2 ± 1.2*</td>
<td>N.D.</td>
<td>25.6 ± 2.3</td>
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</table>
Glucose, Ru = Rutinose, Glc = Glucuronic acid, HE = Hydroxyethyl. * P < 0.05, ** P < 0.01. The enzyme release in control tubes was 28.9 ± 1.7 and 67.5 ± 1.9 (mean ± SE, n = 30) % of the total enzyme content in cells, for β-glucuronidase and lysozyme, respectively.

The flavonols fisetin, kaempferol and quercetagetin-7-O-glucoside, the flavones chrysin, apigenin and luteolin, as well as amentoflavone and to a very reduced extent, naringenin, significantly inhibited arachidonic acid release (Fig. 2). The reference compound, quercetin needed a concentration higher than those able to inhibit purified PLA₂ activity of different origins (Fawzy et al., 1988). We also found a significant correlation between inhibition of β-glucuronidase secretion and of arachidonic acid release (r = 0.73, P < 0.001), as well as between inhibition of lysozyme secretion and of arachidonic acid release (r = 0.82, P < 0.001), for the series of flavonoids studied.

3-Hydroxyflavone was the only compound which exhibited a biphasic effect, since it inhibited β-glucuronidase release without influencing lysozyme release, but at higher concentrations it stimulated degranulation in a significant manner (103.2 ± 2.1 and 54.2 ± 6.5% increase at 10⁻⁴ M, for β-glucuronidase and lysozyme release, respectively, P < 0.01). In another set of experiments carried out in the absence of any stimulus, 3-hydroxyflavone dose-dependently increased β-glucuronidase and lysozyme release, with 282.8 ± 1.5 and 139.5 ± 12.4% increase respectively (P < 0.01), at the concentration of 10⁻⁴ M. Concentrations causing approximately 50% increase with respect to blank samples (without flavonoid) were 7.0 × 10⁻⁵ M and 3.0 × 10⁻⁵ M, respectively.

Fig. 1. Regression lines of amentoflavone (log. μM concentrations) for inhibition of β-glucuronidase (squares) and lysozyme (triangles) release. All points were statistically significant at least at P < 0.05, except for the two lower concentrations of amentoflavone on lysozyme release.

Fig. 2. Effect of flavonoids tested on arachidonic acid release. C = chrysin, A = apigenin, L = luteolin, F = fisetin, Q = Quercetin, QG = quercetagetin-7-O-glucoside, K = kaempferol, N = naringenin and AF = amentoflavone. All results were significant at P < 0.01, except for naringenin (P < 0.05).
Discussion

In our experiments lysozyme release levels were higher than β-glucuronidase release levels as reported by some authors in other systems (Ward et al., 1984; Cockcroft and Stutchfield, 1989). This was probably due to a higher sensitivity of specific granules against different stimuli. This fact could explain that some flavonoids have been more effective on azurophilic granules secretion.

Quercetin has been included in our study as a reference compound since its inhibitory effects on lysosomal enzyme release have been demonstrated in a number of systems such as rabbit neutrophils (Bennett et al., 1981; Showell et al., 1981) and human neutrophils (Blackburn et al., 1987) stimulated by FMLP or concanavalin A (Berton et al., 1980). Our results are in accordance with those previously reported for quercetin, apigenin and luteolin on β-glucuronidase release in human neutrophils (Middleton et al., 1987), although we have used a different cell system. We have also obtained a better potency for flavone and a similar effect for 3-hydroxyflavone, with respect to results reported in rabbit neutrophils (Bennett et al., 1981).

Hypolaetin-8-O-glucoside failed to significantly affect lysosomal enzyme secretion. This is not surprising since many glycosides show a low level of activity on in vitro systems. Nevertheless, in an experimental model of inflammation in rats, we previously demonstrated that this flavonoid inhibited β-glucuronidase release (Villar et al., 1987).

The flavonoids able to inhibit lysosomal enzyme release share some structural features. They are polyhydroxylated aglycones of the flavone or flavonol types and the presence of a free hydroxyl group at C4' increases the inhibitory activity (chrysin-apigenin). Other important features are the keto group at 4 position and the C2-C3 double bond. In contrast, the glycosylation (quercetin-rutin, naringenin-naringin) or the introduction of a free hydroxyl at C2' (kaempferol-morin) seems to be detrimental. In summary, the most active compounds were C2-C3 unsaturated, hydroxylated at C7 and C4' and with some additional hydroxyl groups (at C5, C3 or C3'), characteristics included in those found for inhibition of other leukocyte functions such as reactive oxygen species production by human neutrophils stimulated by FMLP (Limasset et al., 1993).

Flavonoids have been less effective on arachidonic acid release than on lysosomal enzyme secretion; nevertheless, the structural features related to their inhibitory activity are similar for both neutrophil responses.

The precise mechanism of flavonoid inhibition of leukocyte functions is unclear. Recently, it has been suggested a possible interaction of the less hydrophilic flavonoids with the lipid environment of the membrane FMLP receptor or with the receptor itself, while compounds with at least four hydroxyl groups would act at a later stage of the activation process and thus they would influence leukocyte responses in a non-specific way (Limasset et al., 1993). However, interaction of polyhydroxylated flavonoids with membranes has been reported (Ratty et al., 1988) and it has been related to the protective effects of these compounds on different cell types (Perrissoud and Testa, 1986). It is interesting to note that the inhibition of PLA2 activity by quercetin has been explained in part by its ability to interact with phospholipidic substrates (Fawzy et al., 1988). In addition, the behaviour of amentoflavone which decreases basal secretion, suggests a non-specific stabilizing action on cell membranes by this flavonoid, while 3-hydroxyflavone could have the opposite effect, partially masked in the presence of stimulus due to some likely interference with the activation pathway.

We have demonstrated that this series of anti-inflammatory flavonoids can affect neutrophil function through mechanisms not attributable to their effects on either the cyclo-oxygenase or lipoxygenase pathway. Flavonoids have not exhibited a high potency on these in vitro tests, nevertheless high doses of flavonoids are usually needed to exert inhibitory effects on in vivo models of inflammation (Villar et al., 1987; Alcaraz and Jiménez, 1988; Ferrándiz and Alcaraz, 1991) where they may achieve a range of concentrations enough to interact with leukocyte functions. Thus, with the limitations derived from an in vitro study, it is likely that quercetagetin-7-O-glucoside, apigenin, fisetin, kaempferol, luteolin, quercetin and especially, amentoflavone may, at least in part, affect the inflammatory response through their action on neutrophils.

Acknowledgements

This work was supported by grant PM90-0126, from C.I.C.Y.T., Spanish Ministerio de Educación y Ciencia.