Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: Analysis of the structure–activity relationship

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1. Introduction

Flavonoids are a group of polyphenolic compounds that are widely found in the plant kingdom. As intrinsic components of fruits, vegetables and beverages such as wine and tea [1], many of the 4000 different flavonoids known to date are part of the regular human diet. Flavonoids are composed of two aromatic rings linked through three carbon atoms that form an oxygenated heterocycle. Variations on the basic structure of flavonoids yield different classes of flavonoids [2]. These structural variations may explain the observed differences in the bioactivity of these related compounds. Four of the major

Article history:
Received 8 May 2006
Accepted 18 July 2006

Keywords:
Flavonoids
Inflammation
Macrophages
Structure–activity analysis
iNOS
TNF

abstract
Flavonoids possess several biological/pharmacological activities including anticancer, antimicrobial, antiviral, anti-inflammatory, immunomodulatory and antioxidant. The aim of this study was to evaluate the effect of flavonoids on macrophage physiology. For this purpose we selected some flavonoids belonging to the most common and abundant groups (flavonols—quercetin and kaempferol; flavones—diosmetin, apigenin, chrysin and luteolin; isoflavones—genistein and daidzein and flavanones—hesperetin). We decided to use primary bone marrow-derived macrophages (BMDM) as cellular model, since they represent a homogenous, non-transformed population of macrophages that can be stimulated in vitro to proliferate by macrophage colony-stimulating factor (M-CSF) or activated by LPS. In this regard, we demonstrated that most of the flavonoids assayed reduce macrophage M-CSF-induced proliferation without affecting cellular viability. Moreover, some flavonoids also inhibit TNFα production as well as iNOS expression and NO production in LPS-activated macrophages, an effect that has been associated with the inhibition of the NF-κB pathway. We also found that luteolin and quercetin are able to stimulate the expression of the anti-inflammatory cytokine IL-10 at low concentrations (<50 μM). Analysis of the structure–activity relationship showed that four hydroxylations at positions 5, 7, 3′ and 4′, together with the double bond at C2–C3 and the position of the B ring at 2, seem to be necessary for the highest anti-inflammatory effect.

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0006-2952/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2006.07.016
subclasses are flavones, flavonols, isoflavones and flavanones. The first two are the most commonly occurring flavonoids in plants, while flavanones are specially abundant in citrus fruits [1,2]. The flavonones daidzein and genistein and the flavones apigenin and luteolin were recently identified as the main flavonoids accumulated in soybean cotyledons [3,4].

Flavonoids possess various biological/pharmacological activities including antioxidant, antitumour, antiangiogenic, anti-inflammatory, antiallergic and antiviral properties [5]. Of these biological activities, the anti-inflammatory capacity of flavonoids has long been used in Chinese medicine and the cosmetic industry as crude plant extracts. Moreover, some flavonoids have been found to inhibit chronic inflammation in several experimental animal models [6–9]. Current clinical approaches to the treatment of inflammation typically focus on the inhibition of pro-inflammatory mediator production, non-steroidal anti-inflammatory drugs like ibuprofen being a good example. Thus, flavonoids are promising anti-inflammatory drugs that warrant a comprehensive evaluation of its clinical potential as a putative new class of anti-inflammatory agents.

The inflammatory response involves the sequential release of mediators and the recruitment of circulating leukocytes, which become activated at the inflammatory site and release further mediators. The persistent accumulation and activation of leukocytes is a hallmark of different chronic diseases, such as inflammatory bowel disease [10], rheumatoid arthritis [11,12] and septic shock [13]. The capacity to proliferate continuously or a reduction of the clearance of activated cells by apoptosis in the inflammatory site also allows the perpetuation of the inflammatory response.

Macrophages perform essential functions in the organism by acting as regulators of homeostasis and as effector cells in infection, wounding and tumor growth [14]. According to the local needs, resident macrophages either continue proliferating in the presence of M-CSF, the specific growth factor for these cells [15], or become activated and perform their specialized functions [16]. Macrophage activation is characterized by a series of biochemical and morphological modifications that allow these cells to perform their professional functions [14,16]. LPS is an outer membrane component of Gram-negative bacteria and a potent activator of monocytes and macrophages. LPS binds to surface toll like receptor 4 (TLR4), triggering the secretion of a variety of inflammatory products, such as tumour necrosis factor-α (TNFα) and interleukin-1β (IL-1β), as well as high amounts of nitric oxide (NO), which contribute to the pathophysiology of septic shock and other immune diseases [17,18]. Production and release of inflammatory mediators and cytokines by LPS depends on inducible gene expression mediated by the activation of different transcription factors, such as NF-κB [19].

There have been several in vitro studies to investigate the inhibitory activity of flavonoids on NO and cytokine production in different macrophage cell lines, such as RAW 264.7 [18,20–23] and J774.1 [24]. However, the use of immortalized cell lines does not allow the observation of physiologic effects on proliferation-differentiation since the biochemical machinery involved in these processes is disturbed. Here we used primary culture of macrophages obtained from bone marrow, since they represent a homogeneous, non-transformed population of macrophages that can be stimulated in vitro to induce proliferation, differentiation or apoptosis, but also activated to analyze the expression of inflammatory cytokines [25].

The goal of the present study was to test the effects on proliferation and survival as well as the anti-inflammatory properties of different flavonoid compounds (Table 1), to study the structure–activity relationship and to explore some mechanistic aspects of the effects observed. Specially, we focused on the NF-κB pathway, because of its well-characterized involvement in the inflammatory response mediated by macrophages. We selected these nine flavonoids because they are the most common in the human diet and each flavonoid is representative of the different substitutions and structural forms typical of the different flavonoid subclasses (see differences in Table 1). In this regard, we have confirmed the previously reported involvement of the NF-κB pathway in the anti-inflammatory effect of flavonoids but, for the first time, we have observed a new potential mechanism exerted by some flavonoids to modulate the inflammatory response such, i.e. the induction of IL-10 expression exerted by luteolin and quercetin.

2. Materials and methods

2.1. Reagents

Flavonoids were purchased from Extrasynthèse (Genay, France), except genistein and luteolin, which were obtained from Sigma (Madrid, Spain), along with all other reagents unless stated otherwise. In some experiments, we used sulfasalazine as a positive control of an anti-inflammatory drug or a NF-κB-activation inhibitor [26,27]. Flavonoids were diluted in DMSO stock solutions prior to addition to cell culture medium. The final concentration of DMSO never exceeded 0.1% (v/v).

2.2. Macrophage culture

BMDM were isolated from 6-week-old Balb/c mice (Laboratory Animal Service of the University of Granada) as previously described [28]. Mice were killed by cervical dislocation, and both femurs were dissected free of adherent tissue. The ends of the bones were cut off, and the marrow tissue was flushed by irrigation with culture medium. The marrow plugs were dispersed by passing a 25-gauge needle through them, and the cells were suspended by vigorous pipetting and washed. Cells were cultured in 150 mm Petri dishes with 40 ml of DMEM containing 20% FBS and 30% L-cell-conditioned medium as a source of macrophage colony-stimulating factor (M-CSF) [25]. Cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere. After 6 days of culture a homogeneous population of adherent macrophages was obtained (> 99% Mac-1⁺; BD Pharmingen, Heidelberg, Germany) (data not shown). To render cells quiescent, when macrophages were 80% confluent they were deprived of L-cell-conditioned medium for 16 h before carrying out the proliferation assay.

2.3. Proliferation assay

Cell proliferation was measured by [³H]-thymidine incorporation as previously described [29], with minor modifications.
Quiescent cells (10^5) were incubated in 24-well plate in the presence or absence of the indicated flavonoids (25, 50 and 100 μM) or vehicle (0.1%, v/v DMSO) for 1 h before the addition of M-CSF (900 U/ml) and were incubated for 24 h. Then, macrophages were pulsed with [3H]-thymidine (1 μCi/well) for 6 h at 37 °C. After this period cells were fixed in ice-cold 70% trichloroacetic acid and solubilized in 1% SDS and 0.3 M NaOH at room temperature. Radioactivity was counted by liquid scintillation counter. [3H]-Thymidine uptake was expressed as the percentage of the maximum uptake (M-CSF alone). All assays were performed in triplicate and the results are expressed as mean ± S.D.

2.4. Determination of TNFα and IL-10 concentration

Cells were cultured in 24-well plate in the presence or absence of different flavonoid concentrations as indicated for 1 h before the stimulation with LPS (10 ng/ml). Supernatants were obtained 24 h after and frozen at −80 °C until ELISA analysis. Cytokine production was measured with commercial murine ELISA kits (Cytosets™, Biosource International, Nivelles, Belgium) following the manufacturer’s protocol.

2.5. Protein extraction and Western blot analysis

Macrophages were washed with PBS and homogenized in cold lysis buffer containing 1% Igepal CA-630, 20 mM HEPES–Na pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 25 mM MgCl₂, 2 mM sodium orthovanadate and freshly added protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, leupeptin, 1,10-phenanthroline). The protein content was measured by the bicinchoninic acid assay [30], using bovine serum albumin as standard. Samples were boiled for 4 min in Laemml问 buffer, then 100 μg (iNOS) or 65 μg (phospho-IκB-α) were separated by 7% and 10% SDS-PAGE, respectively. The nitrocellulose membranes were blocked for at least 1 h at room temperature in Tris-buffered saline–0.1% Tween-20 (TBS-T) containing 5% (w/v) nonfat dry milk and then incubated with TBS-T containing BSA 5% and the primary antibody at 4 °C overnight. The dilutions of antibodies used were: 1:3000 for iNOS (Transduction Laboratories, BD Biosciences, Madrid, Spain) and 1:1500 for phospho-IκB-α (Cell Signaling Technology, Beverly, MA). A primary antibody against α-actin Sigma (Sigma, Madrid) was used as loading control. After three washes of 5 min with TBS-T, peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as secondary antibody. Then, enhanced chemiluminescence (Perkin-Elmer™, Life Sciences, Boston, USA) detection was performed.

2.6. Determination of NO production

NO production was estimated by measuring nitrate/nitrite in the cell culture media. Macrophages were cultured in DMEM without phenol-red (Invitrogen) to avoid interference with the Griess absorbance at 550 nm. Samples were stored at −80 °C until assayed. Nitrate was converted to nitrite with Zea mays nitrate reductase (Calbiochem). Reduced samples were incubated with an equal volume of Griess reagent, and the
absorbance at 550 nm was measured. The total nitrate/nitrite concentration was determined by comparison with a standard curve.

2.7. Analysis of gene expression by RT-PCR

Total cellular RNA was isolated by single-step, guanidium thiocyanate–phenol–chloroform extraction with the TRizol<sup>®</sup> Reagent (Gibco-BRL, USA) according to the manufacturer’s instructions. RNA (2 μg) from each sample was reverse transcribed into complementary DNA (cDNA) using reverse transcriptase (First-Strand cDNA Synthesis Kit, Amersham Biosciences) and following the instructions as indicated. The primer sequences were: IL-10 (forward 5′-TCCTTAATGCAGGACCTTAGG-3′, reverse 5′-GTTCTGGAGCTTATTAAAAT-3′), β-actin (forward 5′-TGGAAATGCTTGCGCATCC-3′, reverse 5′-AAGCGAGCTCAGTACAGTC-3′). The polymerase chain reaction was performed in a 25 μl volume containing 3 μl of RT product (cDNA) and 22 μl PCR master mix: 10× buffer, 1 U Taq DNA polymerase (Biotherm Genecraft, Germany), 0.2 M of each dNTP (Roche, Germany) and 1 μM concentration of each primer. For each primer pair, control experiments were performed to determine the range of cycles in which a given amount of cDNA would be amplified in a linear fashion: 28 cycles for both IL-10 and β-actin. Semi-quantitative analyses of ethidium bromide-stained DNA gels (2% agarose) were performed with Scion Image (Scion Corporation, USA). The data were normalized to the constitutively expressed β-actin gene transcript levels.

2.8. Statistical analysis

All results are expressed as mean ± S.D. Differences among means were tested for statistical significance using a one-way analysis of variance and post hoc least significance test. The concentration–response curves were fitted with a sigmoidal logistic equation using the Origin 5.0 software (Microcal, Northampton, MA), which provided the calculation of the EC<sub>50</sub>. All other statistical analyses were carried out with the Statgraphics 5.0 software package (STSC, MD), with statistical significance set at <i>p</i> < 0.05.

3. Results

3.1. Effects of flavonoids on M-CSF–macrophage proliferation and viability

For this study we used macrophages obtained from bone marrow cultures, primary cells that can proliferate efficiently in response to M-CSF, the main growth factor used by macrophages. To determine the involvement of flavonoid compounds in the response to M-CSF, we used [3H]-thymidine incorporation as an indicator of cell proliferation, since we have demonstrated previously that this is a valid method that correlates with an increase in the number of macrophages [29]. On the basis of these former studies the concentration of 900 U/ml of M-CSF was selected to induce macrophage proliferation. When flavonoids (kaempferol and quercetin) are added to the culture supernatants, macrophage proliferation is inhibited significantly (Fig. 1A). This effect is concentration dependent, and macrophage proliferation is completely inhibited at a concentration of 50 μM of flavonoids. Additionally, the effect of vehicle (0.1% DMSO) was evaluated on the proliferation of macrophages and no inhibitory effect was observed (Fig. 1A).

Moreover, the proliferative response of bone marrow macrophages to M-CSF is markedly reduced in a concentration-dependent manner in the presence of flavones (25 and 50 μM), with diosmetin and luteolin being the most efficient compounds (Fig. 1B). On the other hand, isoflavones have a biochemical pharmacology 72 (2006) 1010–1021

Fig. 1 – Effect of flavonoids on M-CSF-dependent proliferation of BMDM. Macrophages were incubated in the presence of 900 U/ml of M-CSF in 24-well plate with or without flavonoids (25 and 50 μM). (A) Flavonols: kaempferol and quercetin; (B) flavones: apigenin, chrysin, diosmetin and luteolin; (C) isoflavones: daidzein and genistein; and a flavanone: hesperetin. Each assay was performed in triplicate, and the results are represented as mean ± S.D. of the percentage (%) of thymidine uptake in comparison to non-flavonoid treated macrophages (<i>p</i> < 0.05, <i>**p</i> < 0.01).
lower impact on macrophage proliferation (Fig. 1C), since only genistein shows a significant inhibition at the concentration of 50 μM. Finally, treatment of macrophages with hesperetin results in inhibition of proliferation only at the concentration of 100 μM (50% of the control response, Fig. 1C). These results suggest that flavones and flavonoids seem to be more efficient than isoflavones or flavanones as inhibitors of M-CSF-induced macrophage proliferation.

Since previous reports indicate that flavonoids may induce apoptosis in different cultured cells [31–33], we decided to examine the influence of flavonoid compounds on macrophage viability by crystal violet staining. In all flavonoid groups, none of the compounds tested reduces significantly macrophage viability by 25 or 50 % of the compounds tested reduces significantly macrophage viability by crystal violet staining. In all flavonoid groups, none examine the influence of flavonoid compounds on macrophage apoptosis in different cultured cells [31–33], we decided to

3.2. Effect of flavonoids on TNFα release by LPS-activated macrophages

We also determined the effects of flavonoid compounds on macrophage activation induced by LPS. To this end, cultured mouse macrophages were pretreated with flavonoids (25–50 μM) and the release of TNFα to culture medium measured by ELISA. Virtually all flavonoids tested inhibit LPS-induced TNFα secretion with variable success (Table 2). Thus daidzein and chrysin show only a slight effect on TNFα release (<35% inhibition) that did not reach significance in the case of daidzein at any of the tested concentrations, whereas kaempferol, apigenin, diosmetin and hesperetin reduce TNFα release by approximately 40–55% at 50 μM. Quercetin, luteolin and genistein are the three most efficient inhibitors, allowing only for minimal LPS-induced TNFα release (>60% inhibition) (Table 2).

The inhibitory effect of the three most active inhibitors (quercetin, luteolin and genistein) was further analyzed by assaying lower concentrations of these flavonoids on TNFα secretion (Fig. 2). Genistein inhibits progressively TNFα secretion without achieving total blockade, whereas quercetin and luteolin show a more sigmoidal tendency. The EC50 values obtained with luteolin and quercetin were 10.0 ± 0.3 μM, 20.0 ± 0.6 μM (quercetin) and 30.0 ± 0.9 μM (genistein).

Table 2 – Effects of flavonoids on LPS-induced TNFα release in bone marrow-derived macrophages (%inhibition ± S.D.)

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>Concentration (μM)</th>
<th>TNFα (%inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaempferol</td>
<td>25</td>
<td>17.1 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>53.3 ± 3.1</td>
</tr>
<tr>
<td>Quercetin</td>
<td>25</td>
<td>76.1 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>97.5 ± 3.7</td>
</tr>
<tr>
<td>Apigenin</td>
<td>25</td>
<td>11.4 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>41.6 ± 1.5</td>
</tr>
<tr>
<td>Chrysin</td>
<td>25</td>
<td>23.6 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>32.7 ± 2.9</td>
</tr>
<tr>
<td>Diosmetin</td>
<td>25</td>
<td>37.5 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>48.6 ± 2.5</td>
</tr>
<tr>
<td>Luteolin</td>
<td>25</td>
<td>85.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>97.8 ± 1.2</td>
</tr>
<tr>
<td>Daidzein</td>
<td>25</td>
<td>14.1 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>15.5 ± 3.8</td>
</tr>
<tr>
<td>Genistein</td>
<td>25</td>
<td>48.8 ± 5.3</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>63.2 ± 1.5</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>25</td>
<td>24.1 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>41.6 ± 0.9</td>
</tr>
</tbody>
</table>

*p < 0.05; vs. non-flavonoid treated LPS-activated macrophages.

Fig. 2 – Effect of flavonoids: Gen (genistein), Quer (quercetin) and Lut (luteolin) on LPS-induced TNFα release from mouse macrophages. Cells were pretreated for 1 h with vehicle (DMSO: 0.1%, v/v) or the flavonoid (1, 10, 25, 50, 100 μM) and then stimulated with LPS (10 ng/ml) for 24 h. The medium was collected and analyzed by ELISA as described in Section 2. Results are expressed as the mean ± S.D. of percentage of TNFα secretion in comparison to non-flavonoid treated macrophages (n = 3). The EC50 values are 10.0 ± 0.3 μM (luteolin), 20.0 ± 0.6 μM (quercetin) and 30.0 ± 0.9 μM (genistein).

3.3. Effect of flavonoids on iNOS expression and NO release by LPS-activated macrophages

NO is involved in various biological processes including inflammation [34,35]. Since LPS induces the expression of iNOS in macrophages, and the synthesis of iNOS protein correlates with NO production [36], we analyzed the effect of flavonoids on iNOS expression induced by LPS treatment (10 ng/ml) for 6 h (Fig. 3). Our results show that the flavonol quercetin and the flavones apigenin, luteolin and diosmetin downregulate iNOS expression at low concentrations (<50 μM) (Fig. 3), while higher doses (100 μM) of kaempferol (flavonol) or chrysin (flavone) are required to obtain an inhibitory effect (Fig. 3A and B). Finally, the flavanone hesperetin and the isoflavones genistein and daidzein fail to exert any inhibitory effect on iNOS expression even at 100 μM (Fig. 3C and D).

To confirm these results NO release was also measured indirectly by the quantification of nitrite concentration in the
LPS-activated macrophages in presence of the tested flavonoids. As resumed in Table 3, again, the flavonol quercetin and the flavones apigenin, luteolin and diosmetin were able to inhibit the release of macrophages in a significant way at low concentrations (25–50 μM) while the flavonol kampferol only was able to do it at 50 μM concentration. No effect was observed by the other flavonoids tested on NO production (Table 3).

3.4. Effect of flavonoids on LPS-induced IκB-α phosphorylation

Because both iNOS and TNFα are genes regulated by the NF-κB transcription factor [37,38], we decided to study the implication of this pathway as a potential mechanism mediating the effect of the selected flavonoids. Under quiescent conditions, NF-κB is sequestered in the cytosol bound to the inhibitory protein IκB-α. Exposure of cells to LPS triggers phosphorylation cascades that ultimately lead to phosphorylation and degradation of IκB-α. Once IκB-α dissociates from the complex, NF-κB translocates into the nucleus where binding to specific DNA motifs in the promoter region occurs, leading to increased gene transcription. First, to determine the involvement of IκB-α phosphorylation in LPS macrophage activation, we used the NF-κB inhibitor sulfasalazine. Exposure of macrophages to LPS (10 ng/ml) increases IκB-α phosphorylation on serine 32 as determined by Western blot (Fig. 4A). Phosphorylation of IκB-α by LPS is partially abolished in the cells preincubated with 100 μM of sulfasalazine (Fig. 4B). Moreover, the treatment with sulfasalazine also inhibits iNOS expression after 6 h of LPS stimulation (Fig. 4C).

Pretreatment of the cells with quercetin reduces IκB-α phosphorylation significantly at 10 μM and specially at 50 μM.
Fig. 4 – Effect of sulfasalazine treatment in LPS-activated macrophages. (A) LPS-induced IκB-α phosphorylation in mouse macrophages. Quiescent cells were stimulated with LPS (10 ng/ml) at different time points. Total cell lysates were processed by SDS-PAGE and membranes blotted with an IκB-α-P antibody. (B) Sulf (sulfasalazine) inhibits LPS-induced IκB-α phosphorylation at 100 μM. Sulfasalazine was added 1 h before cells were stimulated with LPS (10 ng/ml) for 10 min. (C) Sulfasalazine 100 μM totally inhibits iNOS expression in mouse macrophages. Densitometric analysis of the western blots was performed and the values obtained are represented in the figure as the normalized band intensity (IκB-α-P or iNOS/actin) and referred as the LPS-stimulated value (1.00). These results represent one of three independent experiments with similar results.

Fig. 5 – Effect of the flavonols quercetin and kaempferol (A), the flavones apigenin, chrysin, diosmetin and luteolin (B), the isoflavone genistein (C) and the flavanone hesperetin (D) on LPS-induced IκB-α phosphorylation in mouse macrophages. Cells were pretreated with the flavonoids for 1 h and then stimulated with LPS (10 ng/ml) for 10 min. The cellular lysates were processed and phosphorylated IκB-α levels analyzed by Western blot. Densitometric analysis of the western blots was performed and the values obtained are represented in the figure as the normalized band intensity (IκB-α-P/actin) and referred as the LPS-stimulated value (1.00). These experiments were performed in triplicate with similar results.

3.5. Luteolin and quercetin induce IL-10 secretion by LPS-activated macrophages

Finally, because the inflammatory process may be inhibited also by the production of immunomodulatory cytokines such as IL-10 or TGF-β [39,40], the effect of the most active flavonoids on IL-10 secretion was investigated. To this end macrophages, which are the main source of the anti-inflammatory cytokine IL-10 [41], were pretreated with the test compounds and then activated with LPS. The inhibitor of NF-κB sulfasalazine was also included as a reference drug (Fig. 6A). Macrophages express IL-10 mRNA after 6 h of LPS stimulation (Fig. 6). While the control drug sulfasalazine does not change IL-10 expression at 100 μM, the addition of apigenin (10, 25 and 50 μM) (Fig. 6) to the cell media before LPS stimulation inhibits mRNA expression in a concentration-dependent manner. This was the case with most other...
flavonoids tested, although to different degrees (data not shown). Conversely, quercetin and luteolin increase IL-10 mRNA levels at low concentrations (1–10 μM) (Fig. 6B). This increase is also observed at the protein level (Fig. 7). However, the effect of both flavonoids disappears at higher concentrations, and luteolin actually inhibits IL-10 secretion at 100 μM (Fig. 7).

4. Discussion

Primary macrophages proliferate in the presence of a specific growth factor, M-CSF, and when they become activated by stimuli such as LPS, they stop proliferating and adopt an activated phenotype, characterized by the expression of early cytokines, such as TNFα and IL-1β, and NO production, as well as distinct morphological changes [42]. Moreover, activated macrophages exhibit a delayed secretion of the anti-inflammatory cytokine IL-10, which has the potential to inhibit the immune response in vivo [43]. We decided to use primary cultures of macrophages as experimental system because they constitute a better model than immortalized cell lines for the characterization of the mechanisms involved in macrophage proliferation, survival and activation [42].

This report describes the effect of several groups of flavonoids in these different macrophage functions. The selection of the flavonoids assayed in this work was based on their prominent presence in foodstuff and hence in the human diet, as well as on their chemical diversity, which allows us to delineate the structural determinants involved in their pharmacological activity (Tables 1 and 4). Thus four different groups were included in the study: flavonols (kaempferol, quercetin), flavones (apigenin, chrysin, diosmetin and luteolin), isoflavones (daidzein, genistein) and a flavanone (hesperetin).

Most of the flavonoids tested have significant antiproliferative effects on M-CSF-activated macrophages, but flavones and flavonols are clearly more efficient than isoflavones or flavanones in this regard. In this case the iso position of the B ring or the absence of a double bond at C2-C3 seems to be correlated negatively with the antiproliferative activity among the compounds tested (Table 4). M-CSF initiates a mitogenic response by binding to its receptor and thereby activating the receptor’s intrinsic tyrosine kinase activity and initiating signaling via multiple effector-mediated pathways [15]. Since some of the

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**Fig. 6** – IL-10 mRNA expression in macrophages treated with apigenin, sulfasalazine (A), quercetin or luteolin (B). Expression was assessed by semi-quantitative RT-PCR. Densitometric analysis of the PCR bands was performed and the values obtained are represented in the figure as the normalized band intensity (IL-10/actin) and referred as the LPS-stimulated value (1.00). The gels shown are representative of at least triplicate experiments.

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**Fig. 7** – Effects of quercetin, luteolin and sulfasalazine on LPS-induced IL-10 secretion. Cells were pretreated with vehicle (DMSO: 0.1%, v/v) (not shown) or the indicated flavonoid (A) and sulfasalazine (B). After 1 h, macrophages were incubated with LPS (10 ng/ml) for 24 h and medium was collected and analyzed as described in Section 2. Experiments were performed in triplicate and the results are expressed as the mean ± S.D. of IL-10 secretion ( *p < 0.05, **p < 0.01; respect to control LPS without flavonoid).
flavonoids used in this work are tyrosine kinase inhibitors, it is likely that this property may account for the antiproliferative effects [44]. The NF-κB pathway is involved in other cellular functions in addition to its proinflammatory actions, including cellular survival and proliferation [45]. However, in our experimental model we can discard this possibility because although flavonoids inhibit the M-CSF–macrophage proliferation, they do not affect the viability of these cells at the concentrations used (data not shown). Moreover, NF-κB does not seem to play a role in macrophage proliferation, since sulfasalazine, at the concentration used, does not inhibit M-CSF–macrophage proliferation (data not shown).

When we analyzed the effects of flavonoids on macrophage activation, we observed different responses depending on the activation marker analyzed (TNFα and iNOS/NO). Thus the flavonoids with 3’ and 4’ hydroxylation (quercetin and luteolin) show the highest degree of inhibition of TNFα, followed by those with only one hydroxyl group on the B ring (genistein, kaempferol, apigenin, diosmetin and hesperetin), regardless of the presence of a double bond in C2–C3, 3’-hydroxylation or iso position of the B ring. Absence of hydroxyl groups in the B ring virtually abolishes this inhibitory activity. On the other hand, when the effects on iNOS and NO release are considered, the flavonoids differ significantly, since isoflavones and flavanones are essentially ineffective, pointing to a determinant role of the position of the B ring and the saturation of the C2–C3 bond. Although Western blotting is only a semi-quantitative technique, flavones appear to be more active than flavonoids. Moreover, NO quantification confirmed the results obtained with the iNOS expression analysis. Among flavones, at least three hydroxyl groups are required to exert a substantial effect (apigenin, luteolin, diosmetin), while flavonoids seemingly require an extra hydroxy (quercetin). Similar findings for these flavonoids have been described in LPS-treated RAW 264.7 cells [18,46]. However, the structural requirements are different from those corresponding to inhibition of antigen-triggered proliferative response in murine and human T cells, in which flavones are more active than flavonols and flavanones [47]. In our experimental model we could not make this generalization regarding flavonoid groups, since flavonols such as quercetin and flavones such as luteolin were equally active. Of note, our results do not support the relevance of the 3-OH (Table 4), which has been previously found to play an important role in other flavonoid properties such as antioxidative activity (see below) or the inhibition of DNA topoisomerases described by Constantinou et al [48].

Although the signaling pathways that lead to induction of both iNOS and TNFα are very similar, a number of differences have been described which may account for the observed differences [27,49]. LPS signaling in macrophages involves a series of phosphorylation events leading to transcription factor activation and increased cytokine and iNOS production. Some of the pathways implicated comprise those involving the Src-family tyrosine kinases, the serine/threonine kinases protein kinase A and C, mitogen-activated protein kinase (MAPKs) and protein kinase B/Akt, and the NF-κB pathway. Certain flavonoids have been reported previously to inhibit protein kinase C [50], tyrosine kinase [51], and phospholipases A2 and C [52,53]. Another possible mechanism includes a downregulation of iNOS indirectly by inhibition of eicosanoid production through the COX/LOX pathway [54]. We have focused on the effect of flavonoids on the NF-κB pathway. Other authors have previously documented the inhibition of IκB-α phosphorylation by flavonoids [51]. However, it should be noted that the correlation between interference with the NF-κB pathway and downregulation of macrophage activation is not perfect, and it correlates better with iNOS expression than with TNFα secretion. This is the case also for sulfasalazine, an NF-κB inhibitor, since it results in full inhibition of iNOS expression but only a modest downregulation of TNFα production (data not shown). In addition, some flavonoids (quercetin, luteolin and genistein) are more effective than sulfasalazine in inhibiting TNFα production. Hence there must be additional pathways involved in the mechanism of action of flavonoids. The mitogen-activated protein kinases (MAPK) family members are a group of serine/threonine kinases that become activated downstream of TLR4 ligation by LPS in

### Table 4 – Overview of flavonoid effects on macrophage physiology and SAR analysis

<table>
<thead>
<tr>
<th>Group</th>
<th>Flavonoid</th>
<th>Prolif</th>
<th>TNFα</th>
<th>iNOS/NO</th>
<th>IκB-α-P</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonols</td>
<td>Kaempferol</td>
<td>+++</td>
<td>++</td>
<td>±/+</td>
<td>±</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>+++</td>
<td>++++</td>
<td>+++/+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Flavones</td>
<td>Apigenin</td>
<td>+</td>
<td>++</td>
<td>++/++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Chrysins</td>
<td>+</td>
<td>+</td>
<td>+/±</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Luteolin</td>
<td>+++</td>
<td>++++</td>
<td>+++/+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Diosmetin</td>
<td>+++</td>
<td>++++</td>
<td>+/+++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Daidzein</td>
<td>±</td>
<td>±</td>
<td>±/±</td>
<td>±</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Genistein</td>
<td>+</td>
<td>++</td>
<td>±/±</td>
<td>±</td>
<td>–</td>
</tr>
<tr>
<td>Flavanones</td>
<td>Hesperetin</td>
<td>+</td>
<td>++</td>
<td>±/±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Structural motives</td>
<td>2-B ring</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C2=C3</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3’,4’-OH</td>
<td>?</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>N’-OH</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3-OH</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>?</td>
<td>±</td>
</tr>
</tbody>
</table>

The magnitude of the effect is classified semi-quantitatively based on maximal effect. Inhibition is shown as +/++++ except for the effect of IL-10, where ++ denotes enhancement and – denotes inhibition. Absence of effect is shown as ±. The contribution of the structural motives is shown as + (positive), – (negative), ± (irrelevant) or ? (unknown).
macrophages. Many studies have demonstrated that inhibition of extracellular signal-regulated (ERK)-1/2, c-Jun N-terminal Kinase (JNK) and p38 activation affects LPS-stimulated TNFα production in macrophages [21,49,55]. Studies are underway to determine the possible effect of flavonoids at this level.

Finally, it is well established that the inflammatory response may be resolved through the clearance of the inflammatory cells or through the release of endogenous anti-inflammatory mediators such as IL-10 and TGF-β [56,57], we decided to analyze the effect of flavonoids in IL-10 secretion induced by LPS in macrophages. We have demonstrated for the first time to our knowledge that luteolin and quercetin stimulate LPS-induced IL-10 secretion. It is interesting to note that this effect vanishes at higher concentrations. This may be due to post-transcriptional effects of the flavonoids, since it is well established that IL-10 can be regulated by post-transcriptional mechanisms in macrophages [58]. Alternatively, flavonoids may be acting as prooxidative molecules at high concentrations. The differential effect observed with these compounds compared with apigenin and the other flavonoids tested constitutes additional evidence that NF-κB is not the only signaling pathway affected by flavonoids. The possible effect of flavonoids on p38 MAPK, which is involved in the regulation of IL-10 expression, is being studied [59].

Since flavonoids are particularly known for their antioxidative/radical scavenging properties, which may be partly related to their anti-inflammatory actions and specifically with inhibition of the NF-κB pathway, it is interesting to consider this aspect in relation to the observed effects in this study. The main determinants of antioxidative capacity have been classically considered to be the presence of a catechol group in the B ring, the presence of a 3-OH group, and the C2–C3 double bond [60]. Thus there is a partial correlation with the structure–activity relationship of the flavonoids as inhibitors of iNOS and TNFα induction in macrophages, since quercetin and luteolin are the most active compounds in both categories. However, kaempferol is an inferior inhibitor despite having substantial antioxidative activity, while apigenin is a poor antioxidant with high in vitro anti-inflammatory effects [61]. Thus, we suggest that antioxidative properties probably do not play a primary role in these flavonoid mechanisms.

Considered together, our results suggest that luteolin and quercetin may be the best flavonoid candidates to provide anti-inflammatory relief in vivo because of their inhibitory effects on TNFα and iNOS expression coupled to the enhancement of IL-10 release. However, the pharmacokinetic characteristics of flavonoids have to be considered before these agents may be used successfully in vivo. Most flavonoids are found glycosylated in nature and cannot be absorbed by the intestinal epithelium in that form [62]. Enzymatic hydrolysis of the glycosides by the intestinal flora yields free flavonoids that are absorbed efficiently. Thus it is likely that these flavonoids must be administered in glycosidic form in order to be applied as anti-inflammatory agents.

Acknowledgements

This work was supported by grants from the Spanish Ministry of Science and Technology (SAF2002-02592 and SAF2005-03199), from the Instituto de Salud Carlos III (PI051625) and from the Junta de Andalucía (CTS-164). MC is a member of the “Juan de la Cierva” program, Ministerio de Ciencia y Tecnología, Spain. IB is a recipient of a pre-doctoral fellowship from Fundación Ramón Areces, Madrid, Spain. EB is a recipient of a pre-doctoral fellowship from the Ministerio de Ciencia y Tecnología, Spain.

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