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Effect of flavonoids on rat splenocytes, a structure–activity relationship study

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ARTICLE INFO

Article history:
Received 31 March 2008
Accepted 3 June 2008

Keywords:
Lymphocyte
Flavonoid
Viability
Toxicity
Proliferation

ABSTRACT

Flavonoids are polyphenols frequently consumed in the diet which have been suggested to exert a number of beneficial actions on human health, including intestinal anti-inflammatory activity. Their properties have been studied in numerous cell types, but little is known about their effect on leukocyte biology. We have selected 9 flavonoids (extended to 14 flavonoids plus the related polyphenol resveratrol in some cases) with different structural features to characterize their effects on leukocyte viability, proliferation, and expression of cyclooxygenase 2 (EC 1.14.99.1), inducible nitric oxide synthase (iNOS, EC 1.14.13.39) and proinflammatory cytokines (TNF-α, IFN-γ, IL-2), as well as to elucidate the structural requirements in each case. Quiescent and concanavalin A-stimulated rat splenocytes were used as a model. Flavonoids (50 μM) had a dramatic inhibitory effect on cytokine secretion. Inducible nitric oxide synthase expression was also blocked largely by some flavonoids, especially quercetin, luteolin and apigenin, while cyclooxygenase 2 was downregulated only by apigenin, diosmetin and quercetin. Apigenin, luteolin, genistein and quercetin had substantial cytotoxic/proapoptotic effects, while chrysin, daidzein, hesperetin and kaempferol did not reduce cell viability. In contrast, all flavonoids had powerful antiproliferative effects. However, none of the compounds activated caspase 3 (EC 3.4.22.56), but actually lowered caspase 3 activation and expression in concanavalin A-stimulated cells. The activity of the quercetin metabolite isorhamnetin was generally lower than that of the parent compound. We conclude that flavonoids have powerful effects on lymphocytes with distinct structural requirements that may contribute to their intestinal anti-inflammatory activity. The bioactivity of orally administered flavonoids may be dampened by biotransformation *in vivo*, particularly in extraintestinal sites.

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Abbreviations: ConA, concanavalin A; COX2, cyclooxygenase 2; DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorting; IFN-γ, interferon gamma; IL, interleukin; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor alpha.

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doi:10.1016/j.bcp.2008.06.001

1. Introduction

Flavonoids are naturally occurring phenolic compounds found in plants and commonly consumed in the human diet [1]. Flavonoids occur in foods primarily as glycosides and polymers which are degraded to variable extents in the digestive tract [2]. The defining structure of flavonoids consists of two fused rings, an aromatic A ring and an oxygen-containing heterocyclic C ring attached by carbon–carbon bond to an aromatic B ring (Table 1). Substituents attached to any of the different positions determine the class of flavonoid to which the compound belongs. Flavonoids have been thus classified according to their chemical structure, and they are usually subdivided into the following subgroups: flavanones, flavones, flavonols, isoflavonoids, anthocyanins, and flavans [1].

Table 1 – Structural features of the flavonoids tested

Chemical formula	Name	Substitution				
Flavonols		5	7	3'	4'	Other
	Kaempferol	OH	OH	H	OH	-
	Fisetin	H	OH	OH	OH	-
	Morin	OH	OH	H	OH	2'-OH
	Myricetin	OH	OH	OH	OH	5'-OH
	Quercetin	OH	OH	OH	OH	-
	Rutin	OH	OH	OH	OH	3'-rutinoside
Flavones		5	7	3'	4'	
	Apigenin	OH	OH	H	OH	
	Chrysin	OH	OH	H	H	
	Diosmetin	OH	OH	OH	OCH_3	
	Luteolin	OH	OH	OH	OH	
Isoflavones		5	7	3'	4'	
	Daidzein	H	OH	H	OH	
	Genistein	OH	OH	H	OH	
Flavanones		5	7	3'	4'	
	Hesperetin	OH	OH	OH	OCH_3	
	Naringenin	OH	OH	H	OH	
Related compound		5	3	3'	4'	
	Resveratrol	OH	OH	H	OH	

Flavonoids have been shown to display a number of interesting biological activities such as antioxidant [3,4], antiviral, antifungal [5], anticancerous [6,7], antiangiogenic [8] and anti-inflammatory activity [9,10]. The biological effects of flavonoids have been classically related to their ability to inhibit enzymes and/or to their antioxidant properties, but recent reports demonstrate that these compounds appear to strongly influence cell-signaling pathways and gene expression with relevance to, among others, cancer, heart disease and inflammatory disorders. Several studies suggest that flavonoid properties may be mediated through the regulation of the MAP kinase [11,12] and NF-κB pathways [13,14]. We [15–19] and other authors [20–23] have established the intestinal anti-inflammatory activity of several flavonoids. These effects have been ascribed to different actions on signal transduction pathways in a number of cell types, including enterocytes, macrophages and mast cells [24–29], while relatively few studies have focused on the flavonoid modulation of lymphocyte biology [30]. Thus epigallocatechin-3-gallate, one of the most important flavonoid-type compounds contained in green tea, can suppress human peripheral blood mononuclear cell and murine splenocyte activation, resulting in lower proliferation and diminished cytokine release [31]. Other compounds can regulate the activity of proinflammatory mediators resulting in pharmacological actions, like a reduction of cardiac graft disease by tea catechins [32]. Quercetin, included in our study, has been suggested to be useful in experimental allergic encephalomyelitis because it blocks IL-2 signaling and the subsequent Th1 differentiation [33]. Likewise, andrograpanin reduces lymphocyte and monocyte chemotaxis induced by SDF-1α, a stromal chemokine [34]. On the other hand, wogonin can reduce the immunosupressing but not the anti-inflammatory effect of glucocorticoids by inhibiting lymphocyte apoptosis [35]. Therefore, flavonoids have been reported to exert both inhibitory and stimulating effects on lymphocyte function/survival. It should be noted that many of these studies have been carried out in tumor cell lines, and thus they may not reflect the activity on normal cells. Moreover, to the best of our knowledge there is no previous study addressing the effect of flavonoids on lymphocytes and the structural requirements for the activity. To this end we have used quiescent and concanavalin A-stimulated rat splenocytes as an experimental model. Flavonoids belonging to four different structural families, plus the related polyphenol resveratrol, were tested for effects on cell proliferation and activation, cytokine release and apoptosis, with the ultimate aim of defining the structure–activity relationships.

2. Materials and methods

2.1. *Reagents*

Flavonoids were purchased from Sigma–Aldrich (St. Louis, MO), along with all other reagents unless stated otherwise. We performed the experiments initially with nine flavonoids (plus quercetin metabolite isorhamnetin), and later carried out cytokine determinations and WST-1 metabolic assays with five additional flavonoids plus resveratrol in order to gain further insight into the structural requirements. 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), which was without effect by itself (not shown).

2.2. *Animals*

Female Wistar rats (190–220 g) obtained from the Laboratory Animal Service of the University of Granada were housed in makrolon cages, maintained with a 12 h light–dark cycle and fed standard rodent chow (Panlab A04, Panlab, Barcelona, Spain) and water *ad libitum* throughout the experiment. This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and was approved by the Animal Welfare Committee of the University of Granada.

2.3. *Primary splenocytes*

Primary splenocytes were obtained from rats sacrificed by cervical dislocation. Using sterile technique, the spleen was extracted, immersed in culture medium and gently dissected with forceps. The resulting cell suspension was cleared of erythrocytes by either short exposure to lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.3) or density separation in Percoll® (GE Healthcare, Bucks, UK). The cells were washed once and cultured at a cellular density of 10^6 cells/ml with or without concanavalin A (5 μg/ml). The culture medium was RPMI supplemented with 10% (v/v) fetal bovine serum, 100 mg/l streptomycin, 100,000 U/l penicillin and 2.5 mg/l amphotericin B. The cells were maintained at 37 °C in standard culture conditions.

2.4. *Proliferation assay*

Cell proliferation was measured by [^{3}H]-thymidine incorporation as previously described [24], with minor modifications. Splenocytes (10^6 ml^{-1}) were cultured with [^{3}H]-thymidine (1 μCi/well, GE Healthcare, Bucks, UK), in the presence or absence of the indicated flavonoids (50 μM) or vehicle (0.1%, v/v DMSO) for 1 h before the addition of concanavalin A (5 μg/ml) or vehicle (sterile PBS), and were incubated for 24 h. After this period the 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 with a liquid scintillation counter. [^{3}H]-Thymidine uptake is expressed as c.p.m. (mean ± S.E.M.). All assays were performed in triplicate.

2.5. *Viability assays*

Splenocytes were cultured at a concentration of 10^6 cells/ml and incubated with flavonoids in the conditions described above for 24 h and then exposed to 0.4% Trypan blue for 3 min. Several dilutions were used to verify obtained results. Stained and unstained cells were counted in three fields and the results expressed as percent of viable (unstained) cells over the total number of cells. Cell viability was also quantitated with the WST-1 assay. Cells were cultured as described and WST-1

solution (Roche Diagnostics, Basel, Switzerland) was added at a 1:10 (v/v) dilution 18 h after concanavalin A stimulation. WST-1 metabolization was assessed 3 h later by spectrophotometric measurement of the formazan product at 450 nm.

Apoptotic cells were detected by flow cytometry. Briefly, cells were cultured as described and harvested after 24 h, washed in PBS and fixed with 70% ethanol. The samples were gently resuspended in DNA extraction buffer (190 mM Na_2HPO_4, 4 mM citric acid, pH 7.8) and incubated for 10 min at 37 °C. The samples were then centrifuged and resuspended in 40 μg/ml propidium iodide with 10 μg/ml of RNAse. The samples were kept in the dark and at 4 °C until analysis and quantitative analysis of sub-G_1 cells was carried out with a FACS Scan cytometer using the Cell Quest Pro® software (Becton Dickinson, Franklin Lakes, NJ, USA).

2.6. Determination of TNF-α, IFN-γ and IL-2

Splenocytes were cultured as described and at a density of 5×10^5 cells/well. Cell culture medium was collected after 48 h, cleared by centrifugation and frozen at −80 °C until assayed for cytokine content by commercial ELISAs (Biosource Europe, Nivelles, Belgium and Becton Dickinson, Franklin Lakes, NJ, USA). In all the experiments, samples were run in triplicate and results are expressed as cytokine concentration (pg/ml).

2.7. Protein extraction and Western blot analysis

Expression of nitric oxide synthase (iNOS, EC 1.14.13.39), cyclooxygenase (COX-2, EC1.14.99.1), caspase 3 (EC 3.4.22.56), and α-actin were determined by immunoblotting. The cells were homogenized in cold lysis buffer containing 1% Igepal CA-630, 20 mM HEPES (pH 7.5), 10 mM EGTA, 40 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM sodium orthovanadate and freshly added protease inhibitors (phenyl-methylsulfonyl fluoride, aprotinin, leupeptin, 1,10-phenanthroline). The protein content was measured by the bicinchoninic acid assay [36], using bovine serum albumin as standard. Samples were boiled for 4 min in Laemli buffer, and then 40 μg were separated by 7% or 13% SDS-PAGE and transferred to nitrocellulose membranes. Reversible Ponceau staining was used routinely to ensure equal loading (not shown). The 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 5% BSA and the primary antibody at 4 °C overnight. The dilutions of antibodies used were: 1:2500 for iNOS (Transduction Laboratories, Lexington, KY, USA); 1:3000 for COX-2 (Cayman Chemical Company, Ann Arbor, MI, USA); 1:1000 for caspase 3 (MBL, Woburn, MA, USA) and 1:1000 for α-actin (DSHB, University of Iowa, USA). The JLA20 antibody against α-actin was obtained from the Development Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences. After three washes of 5 min with TBS-T, peroxidase-conjugated anti-rabbit or anti-mouse IgG were used as secondary antibodies. Then, enhanced chemiluminescence (PerkinElmer™, Waltham, MA, USA) detection was performed. Band density was quantitated using Scion Image (Scion, Frederick, MA, USA).

2.8. Statistical analysis

Experiments were performed at least three times. All results are expressed as mean ± S.E.M. Differences among means were tested for statistical significance using a one-way analysis of variance and post hoc least significance tests were conducted on predefined pairs. All analyses were carried out with SigmaStat® 2.03 (Jandel Corporation, San Rafael, CA, USA).

3. Results

3.1. ConA-induced COX2 and iNOS expression is regulated by flavonoids

We focused initially on the expression of COX2 and iNOS, since these enzymes are upregulated in parallel with lymphocyte activation, they are usually induced in inflammatory conditions and they have been shown to be lowered by flavonoid treatment *in vivo* [17,37,38]. Under basal conditions iNOS and COX2 were weakly expressed and were not affected by flavonoid treatment (data not shown). Stimulation with concanavalin A (5 μg/ml) led to an increase in the protein levels of both enzymes as measured by Western blot (Fig. 1). Several of the compounds tested had a significant effect. Thus apigenin, luteolin and quercetin fully prevented iNOS induction, while diosmetin and chrysin caused a significant inhibition but were less efficient. Hesperetin, kaempferol and the isoflavones genistein and daidzein had no effect (Fig. 1). Therefore only hydroxyl flavones and flavonols (except kaempferol) were able to inhibit iNOS expression, in contrast

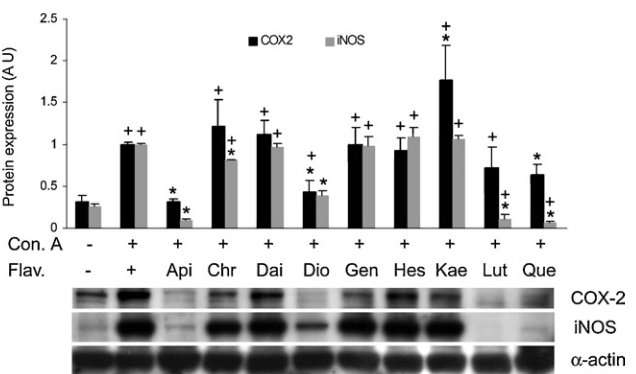
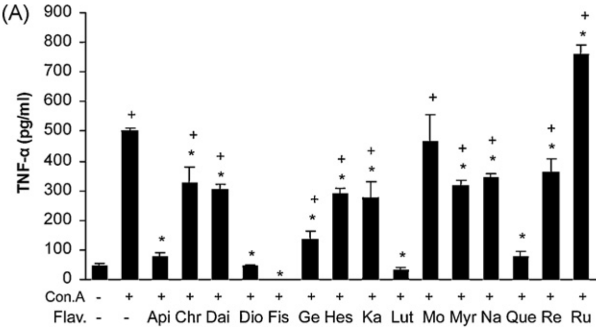
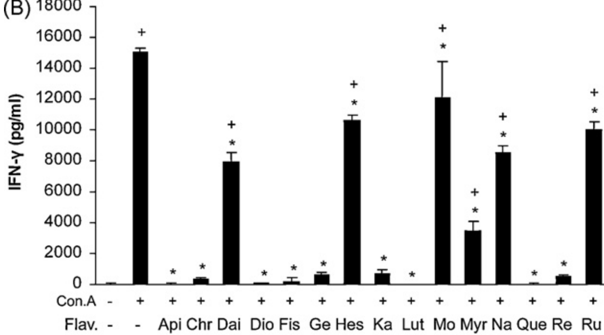


Fig. 1 – Effect of flavonoids on concanavalin A-induced COX2 and iNOS expression. Cells were incubated with Concanavalin A (5 μg/ml), with and without the indicated flavonoids (50 μM) for 24 h. Protein samples were analyzed by Western blot. The density of the bands resulting from three different assays was quantitated using Scion Image and expressed as arbitrary units (AU). Blots are representative of three different experiments. +*p* < 0.05 vs. control and **p* < 0.05 vs. ConA-stimulated cells. Api (apigenin), Chr (chrysin), Dai (daidzein), Dio (diosmetin), Gen (genistein), Hes (hesperetin), Kae (kaempferol), Lut (luteolin), and Que (quercetin).





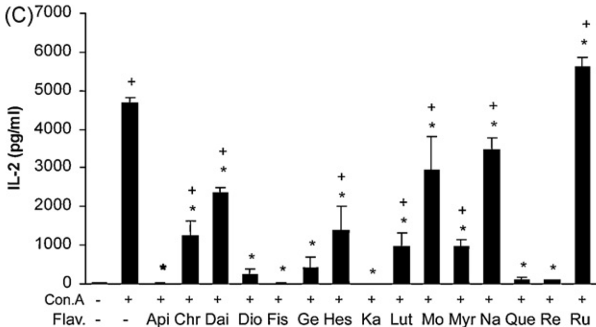


Fig. 2 – Effect of flavonoids on concanavalin A-induced cytokine secretion. Splenocytes were plated in 24-well plates (5×10^5 cells per well) and stimulated with concanavalin A (5 μg/ml) in the presence or absence of flavonoids at 50 μM. After 48 h of incubation, culture medium was collected and frozen at −80 °C until ELISA analysis. Results are expressed as mean ± S.E.M. of cytokine concentration (pg/ml). $^{+}p < 0.05$ vs. control, $^{*}p < 0.05$ vs. ConA-stimulated cells. Cytokine concentration (basal and ConA-stimulated) was 44.1 ± 23.2 and 500.8 ± 21.8 for TNF-α (A), 43.12 ± 55.1 and 15018.1 ± 652.7 for IFN-γ (B), 18.5 ± 25.6 and 4682.9 ± 308.7 for IL-2 (C). Api (apigenin), Chr (chrysin), Dai (daidzein), Dio (diosmetin), Fis (fisetin), Ge (genistein), Hes (hesperetin), Ka (kaempferol), Lut (luteolin), Mor (morin), Myr (myricetin), Na (naringenin), Que (quercetin), Re (resveratrol), and Ru (rutin).

to isoflavones and the flavanone hesperetin. On the other hand, the flavones apigenin and diosmetin, and the flavonol quercetin were the only compounds that reduced COX2 upregulation. Chrysin, genistein and daidzein had no significative effect but, surprisingly, kaempferol-enhanced concanavalin A evoked COX2 induction. Taken together, these data indicate that flavonoids have distinct effects on activated rat splenocytes, depending on the compound considered.

3.2. *Flavonoids downregulate concanavalin A-induced cytokine secretion*

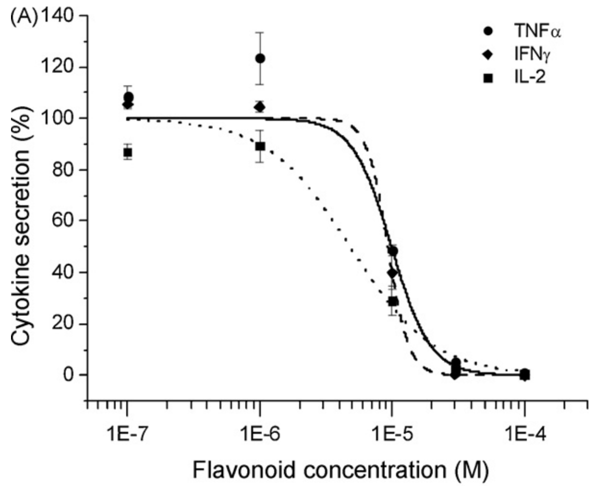
Cytokines play a pivotal role in the development and resolution of inflammatory responses and are essential for lymphocyte regulation of immune function. Because flavonoids were shown to affect splenocyte phenotype we tested the hypothesis that cytokine release is changed by flavonoid treatment as well. We focused on the Th1 prototype cytokines TNF-α, IFN-γ and IL-2, since some flavonoids are capable of treating Th1 inflammatory diseases in preclinical models [28,33]. As described above, there was no detectable effect of any of the compounds tested on quiescent splenocytes (data not shown), which showed low levels of cytokine secretion (Fig. 2). As expected, ligation of the T-cell receptor by concanavalin A brought about a dramatic increase in the release of the three cytokines. Interestingly, all the flavonoids counteracted effectively cytokine production (with the sole exception of morin on TNF-α and rutin on TNF-α and IL-2), although to a different extent. In particular, TNF-α induction was totally abrogated after incubation with quercetin, fisetin, apigenin, diosmetin, luteolin or genistein, while kaempferol, myricetin, daidzein, chrysin, naringenin and hesperetin only inhibited the TNF-α surge by 50% or less. In contrast, IFN-γ levels were virtually normalized to control levels after flavonoid treatment in all cases, except in the case of morin, rutin, daidzein, hesperetin and naringenin, which resulted in partial inhibition. Finally, IL-2 was also reduced dramatically by flavonoids, especially by the flavonols, as well as apigenin, luteolin, diosmetin and genistein, which prevented completely the concanavalin A elicited increase (Fig. 2). Morin, rutin, chrysin, daidzein, naringenin and hesperetin produced a less pronounced inhibition. These data indicate that flavonoids have striking inhibitory effects on cytokine production. We also tested the effect of the flavonoid related polyphenol resveratrol. This compound inhibited IL-2 and IFN-γ completely and TNF-α partially, just like the most effective flavonoids.

In order to better characterize the concentration dependency of flavonoid effects we performed concentration response curves with one of the most active flavonoids, apigenin (Fig. 3). This compound was a slightly more potent inhibitor toward IL-2 than to TNF-α/IFN-γ, and its effects were maximal already at 30 μM in all cases. A concentration response curve was obtained also for daidzein, one of the least active flavonoids, which did not show any further inhibition (not shown).

In principle, these effects may be accounted for by a regulatory action on lymphocytes, by an antiproliferative effect, by cell toxicity/apoptosis, or by a combination of these mechanisms.

3.3. *Flavonoids decrease splenocyte proliferation*

In order to assess the possible antiproliferative effect of flavonoids, a [^{3}H]-thymidine incorporation assay was performed. Addition of 5 μg/ml of concanavalin A led, as expected, to a substantial increase in thymidine uptake,



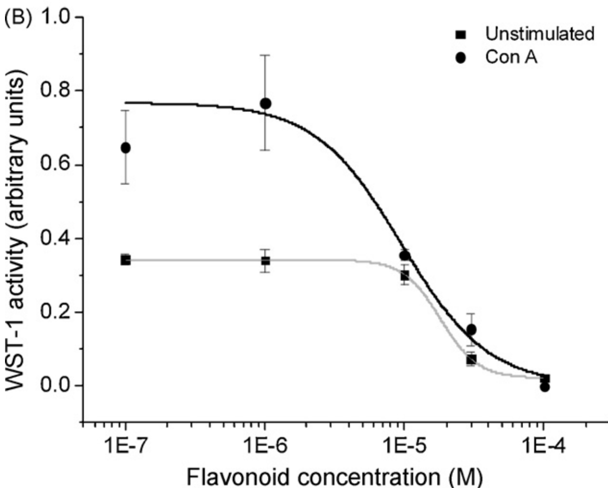
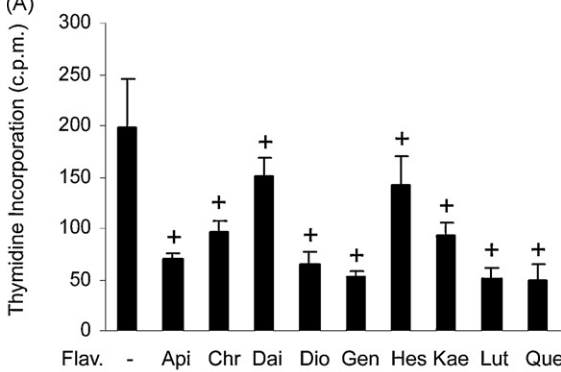


Fig. 3 – Concentration response curve of apigenin: cytokine secretion and viability. Apigenin was used at five different concentrations: 100, 30, 10, 1 and 0.1 μM. (A) Splenocytes were cultured in 24-well plates (5×10^5 cells per well); Con A-stimulated splenocytes were treated with apigenin and cytokine secretion was assessed by ELISA. EC50 values are $9.87 \times 10^{-6} \pm 1.96 \times 10^{-6}$ M for TNF-α, $9.29 \times 10^{-6} \pm 3.01 \times 10^{-6}$ M for IFN-γ and $4.83 \times 10^{-6} \pm 1.35 \times 10^{-6}$ M for IL-2. (B) WST-1 assay was performed under apigenin treatment with or without Con A. Cells were cultured in 96-well plates. WST-1 was added 18 h after Con A stimulation and absorbance at 450 nm was measured 3 h after. EC50 values are $2 \times 10^{-5} \pm 1.72 \times 10^{-6}$ under basal conditions and $9.55 \times 10^{-6} \pm 2.52 \times 10^{-6}$ after Con.A stimulation. Data are expressed as mean ± S.E.M.; $^{+}p < 0.05$ vs. control, $^{*}p < 0.05$ vs. ConA-stimulated cells.



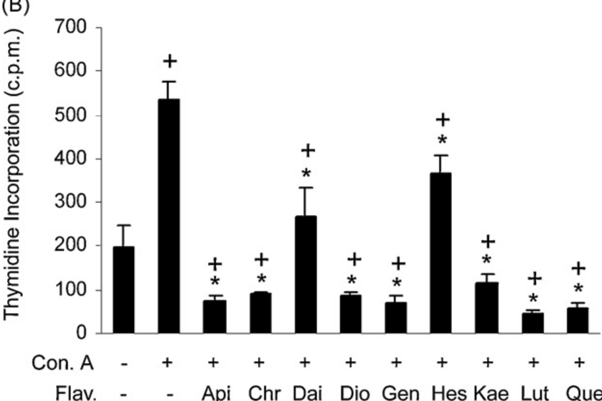
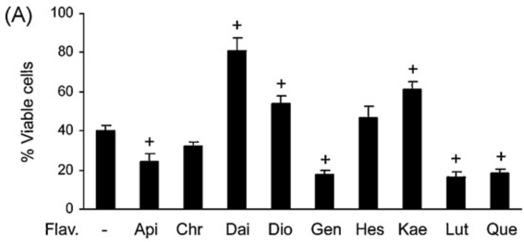


Fig. 4 – Effect of flavonoids on splenocyte proliferation: thymidine incorporation assay. Cells were incubated with (A) flavonoids (50 μM) or (B) Concanavalin A (5 μg/ml), with and without the indicated flavonoids (50 μM). [^{3}H]-Thymidine (1 μCi/well) was added at the same time and uptake was measured 24 h after. Data are expressed as mean ± S.E.M.; $^{+}p < 0.05$ vs. control, $^{*}p < 0.05$ vs. ConA-stimulated cells. Api (apigenin), Chr (chrysin), Dai (daidzein), Dio (diosmetin), Gen (genistein), Hes (hesperetin), Kae (kaempferol), Lut (luteolin), and Que (quercetin).

indicative of cell proliferation (Fig. 4B). Flavonoids showed a very strong antiproliferative effect both on unstimulated (Fig. 4A) and concanavalin A-treated cells (Fig. 4B). In fact, all flavonoids tested reduced cell proliferation to levels far lower (<40%) than those of quiescent splenocytes except daidzein and hesperetin, whose antiproliferative effect was nevertheless considerable. These data suggest that the inhibition of lymphocyte proliferation, which is an integral part of the immune response, is a major effect of flavonoids. It is also possible, however, that flavonoids are toxic or function as proapoptotic stimuli for splenocytes.

3.4. *Effect of flavonoids on cell viability*

We assessed cell viability and toxicity using several approaches. In a first instance cell death was determined by trypan blue staining. Apigenin, genistein, luteolin and quercetin showed a relatively small but significant decrease in lymphocyte viability (Fig. 5A). However, other flavonoids, such as daidzein, diosmetin or kaempferol actually increased cell survival, resulting in a greater percentage of viable cells compared to control cells. In order to further clarify the possible decrease of cell viability exerted by flavonoids, a WST-1 assay was performed. In this test the activity of mitochondrial enzymes, which reduce WST-1 to a soluble colored formazan product, is measured. Thus the absorbance readout is related to the metabolic activity of viable cells.



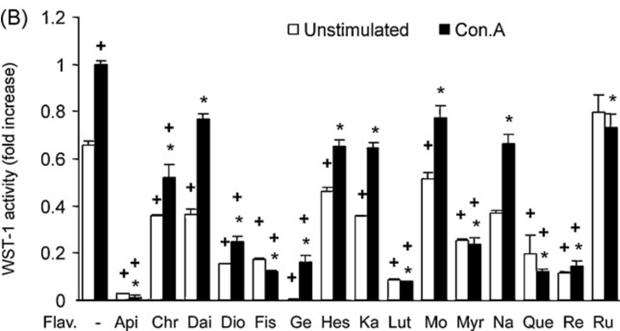


Fig. 5 – Effect of flavonoid on splenocyte viability. (A) Trypan blue assay. 1×10^6 splenocytes were cultured with flavonoids at 50 μM. After 24 h, cells were stained with trypan blue (0.4% in PBS). Viable and not viable cells were counted and results were expressed as % of viable versus total number of cells. (B) WST-1 assay. Cells were plated in 96-well plates and stimulated with or without concanavalin A (5 μg/ml) and with or without flavonoids (50 μM) and 18 h after the WST-1 reduction assay was performed as described. Data are expressed as mean ± S.E.M.; $^+p < 0.05$ vs. control, $^*p < 0.05$ vs. ConA-stimulated cells. Api (apigenin), Chr (chrysin), Dai (daidzein), Dio (diosmetin), Fis (fisetin), Ge (genistein), Hes (hesperetin), Ka (kaempferol), Lut (luteolin), Mor (morin), Myr (myricetin), Na (naringenin), Que (quercetin), Re (resveratrol), and Ru (rutin).

Concanavalin A led to a 1.53-fold increase of absorbance over untreated cells. A strong decrease in reduced WST-1 signal was generally observed with flavonoids (Fig. 5B). This effect in both the basal and concanavalin A-stimulated settings was greater in the case of fisetin, myricetin, quercetin, apigenin, luteolin, diosmetin and genistein, plus the related compound resveratrol. This largely correlates with the results obtained with the trypan blue technique. Morin, kaempferol, rutin, chrysin, daidzein, naringenin and hesperetin were less harmful but still produced a ~50% reduction in cell metabolic activity. Again, a concentration response curve was obtained with apigenin, showing a potency which was similar to that measured for the inhibition of cytokine secretion (Fig. 3).

Next, we monitored whether cell death was due to apoptosis induction by flavonoids using flow cytometry technique. In a physiological situation, the majority of cells are inactive (phase G_0), containing a single DNA copy. Mitotic cells will have a greater amount of DNA and consequently a greater fluorescence emission, while nonviable cells present a lower fluorescent signal due to a lower amount of intact DNA (i.e. fragmented DNA). We have quantified the number of apoptotic cells, i.e. those containing a lower DNA signal vs. the totality of selected cells in the sample (Fig. 6). Unstimulated cells treated with apigenin, luteolin or quercetin showed a marked increase in cell apoptosis, while the other flavonoids did not have significant effects (Fig. 6A and C). Concanavalin A led to a moderate rise in the fraction of nonviable cells, probably due to activation evoked apoptosis, which was not affected significantly by any of the flavonoids (Fig. 6B).

These data, together with those obtained in the cell viability and proliferation assays, suggest that flavonoid exposure results in lymphocyte cell death, at least at 50 μM, especially for some of the compounds, which seems to be due to apoptosis induction in the case of apigenin, luteolin and quercetin.

3.5. *Caspase 3 activation is affected by flavonoid treatment*

In order to further examine the possible effect of flavonoids on lymphocyte apoptosis we assessed the activation of caspase 3, one of the major late apoptotic pathways, by Western blot. As expected, concanavalin A led to a significant activation of caspase 3, with no change in procaspase 3 expression (Fig. 7A). No flavonoid affected caspase 3 expression or activation in basal conditions (data not shown). However, an important effect was observed under concanavalin A stimulation. Thus all flavonoids, except daidzein and hesperetin, abrogated concanavalin A-induced caspase 3 cleavage (Fig. 7A). Daidzein surprisingly increased the active-inactive caspase 3 ratio (2.1 vs. 1.2 in the case of concanavalin A-stimulated cells, $p < 0.05$). Therefore, our results show that most of the flavonoids tested, including those causing cell death, also seem to actually counteract apoptosis by reducing concanavalin A-induced caspase 3 activation (and synthesis).

A simple explanation for these results is that caspase 3 does not appear to be activated by flavonoids because we are looking at the cells at a late time point, so that caspase 3 activation and even cell death may take place earlier. In order to verify the validity of this hypothesis we performed a time course treatment with two selected flavonoids and concanavalin A to study the kinetics of caspase 3 activation under these conditions. Apigenin and daidzein were selected because of their divergent effects. Concanavalin A-evoked caspase 3 activation was observed after 6 h, while neither apigenin nor daidzein led to caspase 3 activation at any time (Fig. 7B). Thus, the effect of flavonoids on quiescent splenocyte viability seems to be related to apoptosis independent of caspase 3 activation and/or to unrelated apoptosis events.

3.6. *Effect of the quercetin metabolite isorhamnetin*

Flavonoids naturally occur in fruits and vegetables predominantly as glycosides, which are extensively hydrolyzed in the intestine, yielding the aglycone polyphenols. However, the latter are subject to covalent modifications within the intestinal mucosa as well as in the liver, so that very small amounts of the aglycone are found in the circulation [39,40]. Thus we decided to study the effect of one of the major metabolites of quercetin, isorhamnetin, on splenocytes and compare it with that of quercetin. Under concanavalin A stimulation, isorhamnetin caused a minimal inhibition of iNOS expression and a mild induction of COX2 (Fig. 8A), while quercetin inhibited iNOS

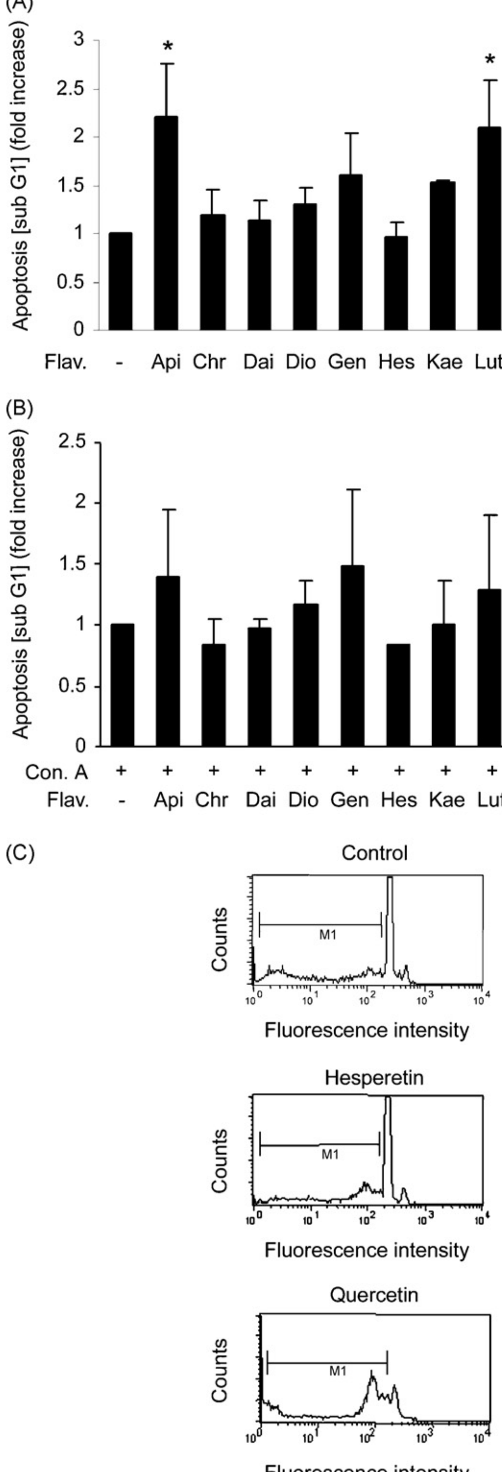
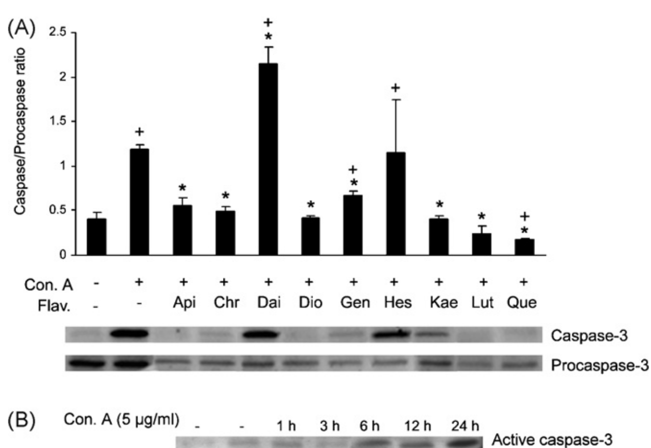


Fig. 6 – Effect of flavonoids on splenocyte apoptosis. Cell cycle and its phases were assessed by FACS. Splenocytes were treated with (A) flavonoids (50 μM) or (B) flavonoids with ConA (5 μg/ml). After 24 h cells were stained with propidium iodide (40 μg/ml). DNA fragmentation (apoptosis) was measured by cell cycle analysis and expressed as percent of cells with sub-G_1 DNA content. (C) An example of fluorescence histograms for control (non-stimulated cells) and two compounds with different effect (hesperetin and quercetin) are shown. Data are expressed as fold increase over the control. Concanavalin A alone induced a 1.43-fold increase of apoptosis in vehicle-treated splenocytes (not shown). Data are expressed as mean ± S.E.M.; $^{*}p < 0.05$ vs. control cells. Api (apigenin), Chr (chrysin), Dai (daidzein), Dio (diosmetin), Gen (genistein), Hes (hesperetin), Kae (kaempferol), Lut (luteolin), and Que (quercetin).



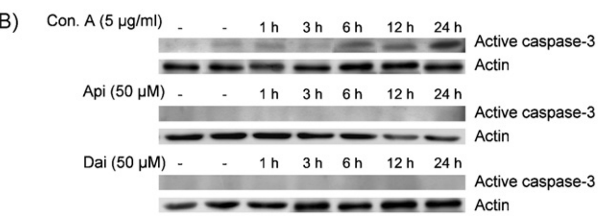


Fig. 7 – Effect of flavonoids on caspase 3 activation. Rat splenocytes were cultured with flavonoids (50 μM), with or without ConA (5 μg/ml). (A) The expression of caspase 3 and procaspase 3 assessed at 24 h by Western blot. (B) The caspase 3 activation was assessed by Western blot at different time points. The density of the bands was quantitated using Scion Image. Results are expressed as caspase:procaspase 3 ratio. A representative blot of three experiments is shown. Data are expressed as mean ± S.E.M.; $^{+}p < 0.05$ vs. control, $^{*}p < 0.05$ vs. ConA-stimulated cells. Api (apigenin), Chr (chrysin), Dai (daidzein), Dio (diosmetin), Gen (genistein), Hes (hesperetin), Kae (kaempferol), Lut (luteolin), and Que (quercetin).

expression completely and showed an inhibitory tendency on COX2 (Fig. 1). Isorhamnetin displayed a similar effect on trypan blue uptake to that of quercetin but, conversely, increased WST-1 reduction by treated cells considerably (Fig. 8B), suggesting a positive effect on splenocyte proliferation. Analysis of cell toxicity by FACS confirmed that isorhamnetin is much less effective than the parent flavonoid in induction of cell apoptosis (Figs. 8C and 6C).

4. Discussion

Our results demonstrate that flavonoids have important effects on splenocytes which depend on their structural features. Flavonoids have been studied at the concentration of 50 μM

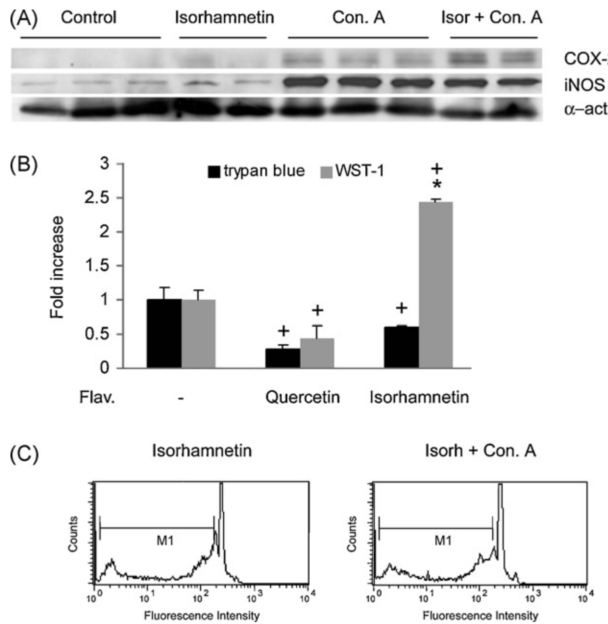


Fig. 8 – Effect of the quercetin metabolite isorhamnetin on splenocyte biology. (A) iNOS and COX expression. iNOS and COX2 expression was measured by Western blot using similar conditions as in Fig. 1. (B) Cell viability. Trypan blue and WST-1 assays were used. Results are expressed as fold increase over untreated cells. Data are expressed as mean ± S.E.M.; $^{+}p < 0.05$ vs. control, $^{*}p < 0.05$ vs. quercetin-treated cells. (C) FACS analysis of cell apoptosis. Cells were examined after 24 h of exposure to isorhamnetin (50 μM), with or without concanavalin A (5 μg/ml). These data are representative of three experiments.

because they have shown bioactivity *in vitro* in these conditions [24,25]. However, additional experiments carried out with one of the most active flavonoids, apigenin, showed that it was clearly active at lower concentrations. Evidence from a number of cell systems indicates that resting cells are generally not affected by flavonoids, while activated cells tend to be more susceptible to their influence, which may alter dramatically the outcome of the activation process [30]. Our experiments are in agreement with this argument since the selected flavonoids did not alter COX2 and iNOS levels or cytokine release in rat splenocytes in quiescent conditions. However, some flavonoids, namely quercetin, apigenin, luteolin and genistein showed toxic/proapoptotic effects in unstimulated cells. Although not tested directly, it is likely that fisetin, myricetin and resveratrol are also cytotoxic, as suggested by complete or near complete WST-1 inhibition. Furthermore, all flavonoids exhibited marked antiproliferative effects in these conditions. On the other hand, under concanavalin A stimulation, flavonoids, particularly quercetin, fisetin, myricetin, luteolin and apigenin, plus resveratrol, had striking effects on splenocytes, which generally consisted in reduced proliferation, iNOS expression, cytokine release and cell viability.

The analysis of the data corresponding to iNOS and COX2 inhibition shows some common and some distinct structural requirements for optimal activity. Thus the position of the B ring in 2 and 4′-hydroxylation favor inhibition in both cases, which is impaired by methoxylation in the case of iNOS. The presence of 3-OH is detrimental for iNOS inhibition (kaempferol vs. apigenin) but may be compensated by additional B ring hydroxylation (quercetin vs. luteolin). Flavonols tend to increase COX2 expression (kaempferol vs. apigenin and isorhamnetin vs. diosmetin), with the exception of quercetin, suggesting that overall hydroxylation may counteract the influence of 3-OH. Interestingly, kaempferol actually had a slight enhancing effect on COX2, suggesting that the predominant effect of the flavonoid 'core' structure is stimulatory rather than inhibitory. The effect of 3′-hydroxylation appears to be detrimental.

Similarly, the requirements for inhibition of cytokine secretion seem to be broadly overlapping, since there was a good correlation in the effect on TNF-α, IFN-γ and IL-2, although the effect of chrysin, rutin, morin, kaempferol and naringenin on TNF-α release was lower, perhaps reflecting simply a less sensitive response in the case of this particular cytokine. In all other cases flavonoids blocked cytokine surge completely (quercetin, fisetin, myricetin, luteolin, apigenin, diosmetin, and genistein), as did resveratrol. TNF-α and IFN-γ have regulatory actions mainly on macrophages, endothelium and stromal cells, while IL-2 is considered primarily a cytokine with autocrine effects on lymphocytes themselves, namely stimulating proliferation by secretion and ligation of specific receptors. In general, there was indication that the structural requirements favoring inhibition include: (1) the 2–3 double bond, since hesperetin and naringenin showed a weaker effect than diosmetin and apigenin (and genistein); (2) 4′-hydroxylation (apigenin vs. chrysin); (3) 3′-hydroxylation (quercetin vs. kaempferol and the fact that myricetin and fisetin retain maximal activity); (4) the absence of 3-OH (apigenin vs. kaempferol, compensated by 3′-OH in the case of quercetin, fisetin and myricetin); (5) the presence of the 5-OH, at least in isoflavones (genistein vs. daidzein). The 2′-OH and the position of the B ring are irrelevant. 3-Glycosylation cancels bioactivity almost completely (rutin vs. quercetin). The flavonoid-related compound resveratrol features hydroxyl groups in positions equivalent to 5,7 and 4′ of the flavonoid structure, and in this regard it is interesting to note that it behaves exactly like the flavonoid showing the closest resemblance, i.e. apigenin.

Although we did not address mechanistic aspects of flavonoid effects, it is interesting to note that iNOS, COX2, IL-2, TNF-α and IFN-γ are regulated to a large extent by the same signaling pathways, such as NF-κB. Because the structural requirements for flavonoid inhibition are quite similar it is likely that this reflects a common mechanism of action.

The fact that flavonoids displayed striking inhibitory effects on cytokine release led us to consider the possibility of cytotoxic/proapoptotic mechanisms, since the cytokine surge is partially dependent on a strong proliferative response. We therefore focused on cell proliferation as well as cell viability, using different experimental assays. We demonstrate in the present work that all the flavonoids under study not only reduced basal proliferation but also suppressed considerably the mitogenic response of rat splenocytes to concanavalin A. The suppression of lymphocyte proliferation induced by mitogens has been previously reported for several flavonoid derivatives [30,41–43]. These studies indicated that

the presence of a C-2,3-double bond was essential for the antiproliferative activity and that the potency of inhibition was dependent on the number and position of hydroxylations of the B-ring. These structural features (C-2,3-double bond and B-ring hydroxylation) are present in all the selected flavonoids in our study except the flavanone hesperetin, which accordingly had a less pronounced effect. The other flavonoid with reduced antiproliferative effect was daidzein, suggesting that the 5-OH is important for this activity, at least in isoflavones. Of note, there is an excellent correlation between inhibition of proliferation and of cytokine secretion, clearly suggesting a mechanistic link. One of the possible mechanisms for reduced proliferation is flavonoid interference with the IL-2 autocrine loop. Interleukin-2 is a proinflammatory mediator capable of supporting long-term T cell proliferation. IL-2 is necessary for the development of T cell immunologic memory and induces the expression of proinflammatory mediators such as IL-1, TNF-α and IFN-γ. Because IL-2 is both required for cell division and increased as a result of proliferation, confirmation of this hypothesis will require additional intervention experiments in which exogenous IL-2 is added to flavonoid-exposed splenocytes. Because concanavalin A is a mitogenic stimulus for lymphocytes the effect of flavonoids may be supposed to be exerted primarily on this cell type, and specifically on T cells. However, splenocytes are a mixed cell population and effects on other cell types, particularly monocytes, cannot be ruled out [22]. Additional experiments will be required to address this issue, for instance using alternative stimuli and/or cell separation techniques.

In addition to their antiproliferative effect, it is clear that some flavonoids are also deleterious to splenocytes. In particular, quercetin, apigenin, luteolin and genistein reduced cell viability as assessed by trypan blue exclusion and had apoptotic effects as quantitated by FACS (not significant in the case of genistein). These four flavonoids, plus diosmetin, also had a dramatic effect in the WST-1 reduction test, which measures the total number of metabolically active cells and thus reflects both proliferation and toxicity. The same result was obtained with fisetin, myricetin and resveratrol, suggesting a similar combination of antiproliferative and cytotoxic effects. In contrast, kaempferol, morin, rutin, chrysin, daidzein, hesperetin and naringenin had a limited impact in the WST-1 test and did not generally cause cell toxicity. In fact, daidzein-, diosmetin- and kaempferol-treated cells showed a lower percent of trypan blue uptake, suggesting increased cell viability. This was not confirmed by the flow cytometry analysis. Of note, when the structural requirements for WST-1 inhibition are considered, it becomes evident that they are exactly the same discussed above for cytokine inhibition. Thus flavones and isoflavones are more active than flavonols, and much more than flavanones, and 4′/3′-hydroxylation favors bioactivity. In accordance with this, there was an almost perfect correlation of potency for inhibition of proliferation/viability and of cytokine secretion in the case of apigenin.

It should be noted that the cytotoxic/proapoptotic effects of flavonoids largely disappear in the presence of concanavalin A stimulation, in contrast with the antiproliferative activity, which remains. Both activities were generally correlated, but diosmetin, chrysin and kaempferol (and possibly morin, rutin and naringenin) were more antiproliferative than cytotoxic.

From a mechanistic point of view, it is not surprising that flavonoids affect cell cycle and viability. Some studies have proven that several polyphenolic compounds can alter cell cycle, affecting DNA replication and decreasing cell viability, via a variety of signaling pathways [30,44,45]. Similarly, there are numerous studies that propose a proapoptotic effect of some flavonoid derivatives on malignant lymphoid cells [46–48]. In fact, there is a growing interest in the role of flavonoids as anticancer agents [6,7,49]. However, even in some of these works, no toxic effect was shown to occur in normal cells. Caspases are the main protein family involved in the execution of apoptosis, caspase 3 and 9 being the most important effector members. We hypothesized that flavonoids could induce apoptosis in rat splenocytes and therefore focused on caspase 3 activation. Surprisingly, flavonoids not only failed to cause protease activation but also actually opposed it when evoked by concanavalin A. We could also rule out an early effect of the polyphenols, indicating that the compounds tested are unlikely to induce apoptosis by caspase 3 activation, although it remains possible that alternative pathways are activated in the case of quercetin, apigenin and luteolin, for instance a mitochondrial-dependent pathway related to the antioxidant or prooxidant effect of flavonoids [50–52].

Whatever the mechanism of flavonoid elicited cell death this phenomenon has possible implications for the anti-inflammatory activity of these compounds. However, the magnitude of the effect calls into question also the possibility that frank toxicity results from *in vivo* administration, especially in the form of flavonoid enriched nutritional supplements or pharmacological treatment. To address this issue we must consider the pharmacokinetic profile of these molecules. Flavonoids access the human body normally by the oral route, majoritarily bound to sugars as glycosides [1]. Glucose-bound flavonols (the most documented flavonoid type) may be absorbed in the small intestine, whereas rhamnoglucosides are not and therefore reach the colon, where they can be hydrolyzed by bacterial enzymes [39,53,54]. The released aglycone moieties can be absorbed effectively from the large intestine or further degraded by bacterial enzymes. Isoflavones may be absorbed efficiently regardless of their glycosylation status [53]. However, whatever the site of absorption little intact flavonoid reaches the systemic circulation because of extensive metabolization, including hydrolysis of absorbed glycosides, conjugation with glucuronic acid or sulfate or *O*-methylation [39]. Biotransformation takes place in the intestinal mucosa as well as in the liver [2]. As a result, free flavonoid aglycones are typically present at very low concentrations in plasma or urine. Plasma concentrations in subjects with a normal diet are characteristically less than 1 μM [55]. Our data suggest that at least some flavonoids have relevant effects on lymphocytes at concentrations slightly over 1 μM. Because flavonoids are extensively metabolized, we also studied the effects of isorhamnetin, one of the major metabolites of the most abundant flavonoid, quercetin, which is also one of the most active according to our data. In fact, the majority of the circulating metabolites of quercetin (91.5%) are reportedly glucurono-sulfo conjugates of isorhamnetin (3′-*O*-methyl quercetin) and of quercetin itself. The remainder (8.5%) is constituted by glucuronides of quercetin and its methoxylated forms [56]. Furthermore, isorhamnetin is the main metabolite

of quercetin found *in vivo* after chronic quercetin administration [57]. Our data indicate that the effects of isorhamnetin are indeed much less prominent than those of quercetin, showing slight changes on iNOS and COX2 expression, a similar reduction in cell viability and enhanced rather than reduced cell proliferation as assessed by WST-1 metabolization. These data are consistent with the fact that diosmetin, which only differs from isorhamnetin in the lack of a 3-OH group, is not cytotoxic and has moderate also iNOS/COX2 modulatory effects. Glycosylation was detrimental for bioactivity in our study, as is generally the case [30], although there are a few exceptions. For instance, Morand et al. have reported the antioxidant properties of conjugated quercetin metabolites and propose that they might modify lymphocyte biology as well [56]. Thus the effect of flavonoids on lymphocytes may be involved in their intestinal anti-inflammatory activity *via* actions restricted to *lamina propria* cells. Any extraintestinal effect of these compounds is likely to be prevented by extensive first pass biotransformation. Enhanced anti-inflammatory and/or toxic effects may occur in case of parenteral administration. Given the role of T cells in inflammatory and autoimmune diseases and the growing list of side effects that drug therapy can produce, flavonoids could be valuable therapeutic agents and full elucidation of their mechanism of action is warranted. Moreover, the upcoming studies should address the importance of flavonoid metabolites in the anti-inflammatory effects observed *in vivo*.

Acknowledgements

This work was supported by grants from the Instituto de Salud Carlos III (PI051625 and PI051651). RLP and IB are recipients of a predoctoral and a postdoctoral fellowship from the Ministerio de Educación y Ciencia, Spain, respectively. ACA-M is funded by the SAF2006-08529 grant and by the Ramon y Cajal program of the Ministerio de Educación y Ciencia, Spain and OMA by the I3P program of the Ministerio de Educación y Ciencia, Spain. The group is member of the Network for Cooperative Research on Membrane Transport Proteins (REIT), co-funded by the Ministerio de Educación y Ciencia, Spain and the European Regional Development Fund (Grant BFU2005-24983-E/BFI). The CIBER-EHD is funded by the Instituto de Salud Carlos III.

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