

In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF- κ B pathway

Mònica Comalada¹, Desirée Camuesco¹, Saleta Sierra², Isabel Ballester¹, Jordi Xaus², Julio Gálvez¹ and Antonio Zarzuelo¹

¹ Department of Pharmacology, University of Granada, Granada, Spain

² Department of Immunology and Animal Sciences, Puleva Biotech, S.A., Granada, Spain

Quercetin is a common antioxidant flavonoid found in vegetables, which is usually present in glycosylated forms, such as quercitrin (3-rhamnosylquercetin). Previous *in vitro* experiments have shown that quercetin exerts a bigger effect than quercitrin in the down-regulation of the inflammatory response. However, such results have not been reproduced in *in vivo* experimental models of intestinal inflammation, in which quercetin did not show beneficial effects while its glycosides, quercitrin or rutin, have demonstrated their effectiveness. In this study, we have reported that the *in vivo* effects of quercitrin in the experimental model of rat colitis induced by dextran sulfate sodium can be mediated by the release of quercetin generated after glycoside's cleavage by the intestinal microbiota. This is supported by the fact that quercetin, but not quercitrin, is able to down-regulate the inflammatory response of bone marrow-derived macrophages *in vitro*. Moreover, we have demonstrated that quercetin inhibits cytokine and inducible nitric oxide synthase expression through inhibition of the NF- κ B pathway without modification of c-Jun N-terminal kinase activity (both *in vitro* and *in vivo*). As a conclusion, our report suggests that quercitrin releases quercetin in order to perform its anti-inflammatory effect which is mediated through the inhibition of the NF- κ B pathway.

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Introduction

Flavonoids comprise a large group of compounds occurring widely throughout the plant kingdom. Daily flavonoid intake (typically present in onion, apple,

grape, wine, herbs and spices) in the human diet is highly variable, with estimations ranging from 23 mg/day [1] to more than 500 mg/day [2]. Flavonoids exert several biological activities, which are mainly related to their ability to inhibit enzymes and/or to their antioxidant properties, and are able to regulate the immune response [3]. These activities may explain the beneficial effects that flavonoid intake exerts in different human pathologies, including hypertension, inflammatory conditions and even cancer [4].

Among flavonoids, quercetin is the most common flavonoid in nature, and it is mainly present as its glycosylated forms such as quercitrin (3-rhamnosylquercetin) or rutoside (3-rhamnosylglucosyl quercetin) [5]. *In vitro* studies have clearly shown that quercetin acts as a potent pleiotropic modulator in several

The last two authors contributed equally to the supervision of this study.

Correspondence: Mònica Comalada, Department of Pharmacology, School of Pharmacy, University of Granada, Campus "La Cartuja" s/n, E-18071 Granada, Spain
e-mail: jxaus@puleva.es

Abbreviations: **IBD:** Inflammatory bowel disease · **BMDM:** Bone marrow-derived macrophages · **M-CSF:** Macrophage colony-stimulating factor · **DAI:** Disease activity index · **DSS:** Dextran sulfate sodium · **MPO:** Myeloperoxidase · **iNOS:** Inducible nitric oxide synthase · **JNK:** c-Jun N-terminal kinase

physiological functions, showing different activities such as anti-proliferative effect in numerous cell lines [6, 7], pro-apoptotic effect in lung carcinoma cell lines [8], and inhibitory effect of osteoclastic differentiation [9]. It is important to note that when the glycosylated forms of quercetin are assayed, there is usually a loss of activity in these effects in comparison with those obtained with the aglycone, due to the presence of the sugar moiety in the flavonoid structure [10]. On the contrary, both glycosides, quercitrin and rutoside, have shown to exert intestinal anti-inflammatory effects in experimental models of rat colitis [11, 12], whereas no clear effects have been demonstrated for the aglycone form (unpublished results). The aim of this study was to clarify this controversy.

Chronic inflammatory bowel disease (IBD), mainly ulcerative colitis and Crohn's disease, is a naturally remitting and recurring condition of the digestive tract. As in other inflammatory processes, IBD is characterized by an up-regulation of the synthesis and release of a variety of pro-inflammatory mediators, such as eicosanoids, platelet activating factor, reactive oxygen and nitrogen metabolites and cytokines, thus influencing mucosal integrity and leading to excessive injury [13]. The cell types involved in the mucosal inflammatory response are similar to those found at systemic inflammatory sites including macrophages [14]. These cells have critical functions in the immune system, acting as regulators of homeostasis and as effector cells in infection, wound healing and tumor growth. However, macrophages do not always play a positive role in the homeostasis of the immune system. Under some circumstances, such as septic shock [15], rheumatoid arthritis [16], atherosclerosis and IBD [17], macrophages have been described to have noxious effects, probably due to the non-regulated and excessive secretion of inflammatory modulators such as reactive metabolites from oxygen or nitrogen and pro-inflammatory cytokines, including TNF- α , being deleterious to intestinal function. In fact, macrophages are considered to be the main source of these pro-inflammatory mediators in IBD, thus actively contributing to the pathology of these intestinal conditions [14, 18].

In the present study, we try to assess the *in vitro* mechanisms involved in the intestinal anti-inflammatory effects of quercetin/quercitrin on macrophage biology. Especially, we focus on the NF- κ B and c-Jun N-terminal kinase (JNK) activation pathways, since it is well established that these pathways participate in the inflammatory response mediated by macrophages [19, 20]. Moreover, we tested the effects of quercitrin in the dextran sulfate sodium (DSS) model of rat colitis and correlated them with the observations obtained with the *in vitro* models. Finally, we analyzed the hydrolysis of quercitrin to yield quercetin by the intestinal microflora

as a key step in the intestinal anti-inflammatory effect of the glycoside. In conclusion, our results give additional information about the mechanism of action of flavonoids in the management of intestinal inflammatory conditions.

Results

The first aim of this work was to address whether quercetin or its glycoside quercitrin regulate some aspects of macrophage biology, such as proliferation or activation. Macrophage colony-stimulating factor (M-CSF) induced the proliferation of quiescent bone marrow-derived macrophages (BMDM) in a dose-dependent manner as measured by [3 H]thymidine incorporation (Fig. 1). This method gives an efficient indication of macrophage proliferation as previously determined [21] and correlates with the number of cells. Quercetin inhibited in a dose-dependent manner the M-CSF-dependent proliferation of these cells. In contrast to quercetin, quercitrin was unable to modify the proliferative response of macrophages, although higher doses (100 μ M) were used (Fig. 1).

The next aim was to study the effect of quercetin/quercitrin on macrophage activation. BMDM expressed high levels of TNF- α protein after 6 h of LPS stimulation (Fig. 2A). LPS also induces the expression of IL-1 β protein, partly as a consequence of TNF- α release [22], and for this reason we detected IL-1 β protein after 12 h of LPS stimulation (Fig. 2A). The addition of quercetin (50 μ M) to the cell media before LPS stimulation partially inhibited both cytokines' expression (Fig. 2A). We did not detect any effect on the protein expression of these cytokines when different concen-

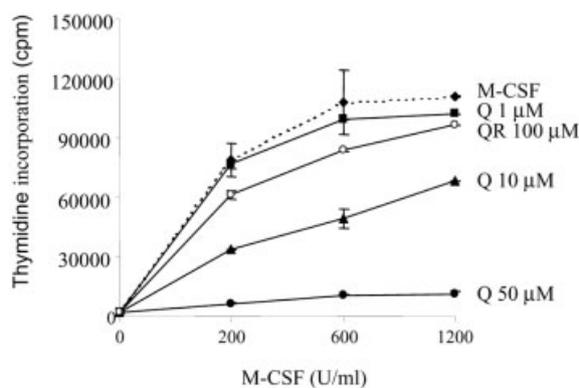


Fig. 1. Quercetin, but not quercitrin, inhibits the M-CSF-dependent proliferation of BMDM. Macrophages (10^5) were incubated in the presence of the indicated amounts of M-CSF in 24-well plates with or without quercetin (Q; 1, 10, 50 μ M) or quercitrin (QR; 100 μ M). Results are represented as means \pm SD of triplicates.

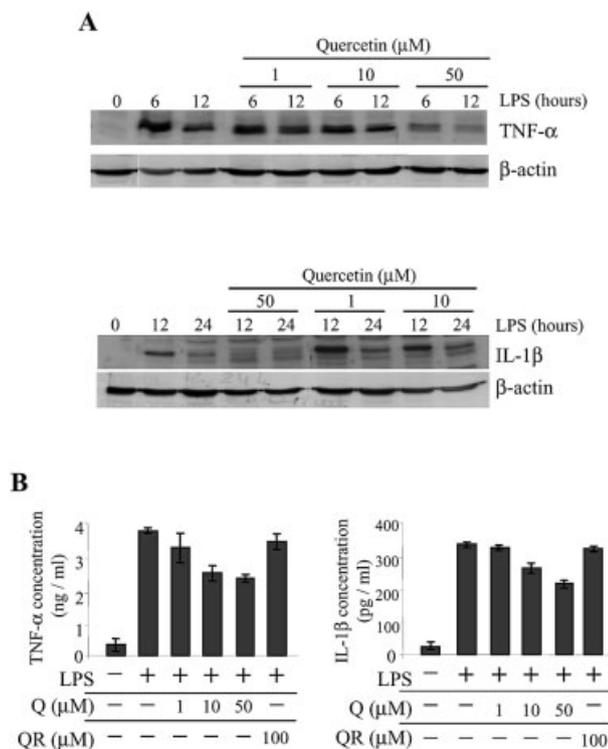


Fig. 2. Quercetin, but not quercitrin, inhibits cytokines induced by LPS in BMDM. (A) Quercetin inhibits TNF- α and IL-1 β protein expression. Cells were incubated with quercetin (1, 10, 50 μ M) for 1 h and then stimulated with LPS (50 ng/ml) for the indicated times. (B) Quercetin reduces TNF- α and IL-1 β secretion. Macrophages (10^5) were cultured and treated as indicated in (A) for 24 h. Concentrations of TNF- α and IL-1 β in the culture supernatants were analyzed by Western blot (A) or ELISA (B). The results of a representative experiment of four independent experiments are shown as means \pm SD of triplicates.

trations of quercitrin were used (data not shown). These results were confirmed by analyzing the TNF- α and IL-1 β secretion by measuring their expression in the cell culture supernatants (Fig. 2B). As in the previous experiment, the pretreatment with quercetin, but not quercitrin, inhibited in a dose-dependent manner the secretion of TNF- α and IL-1 β by almost 40% (Fig. 2B).

Since LPS also induces the expression of inducible nitric oxide synthase (iNOS) in macrophages [23], we analyzed the effect of quercetin/quercitrin on LPS-induced iNOS expression in macrophages. After 6 and 12 h of LPS stimulation, iNOS expression was induced in these cells (Fig. 3). When macrophages were pre-incubated with different concentrations of quercetin (1, 10 and 50 μ M), a dose-dependent inhibition of the LPS-induced iNOS expression was observed (Fig. 3). However, in contrast to quercetin, quercitrin pretreatment (25–100 μ M) was not able to modify the expression of iNOS induced by LPS (data not shown).

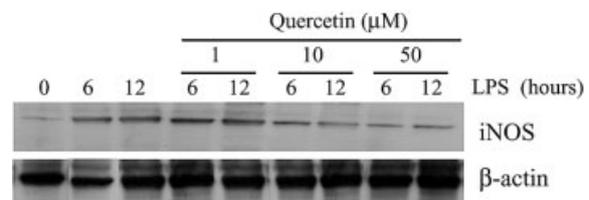


Fig. 3. Quercetin inhibits the iNOS expression induced by LPS in BMDM. Macrophages were incubated with quercetin (1, 10, 50 μ M) for 1 h and then stimulated with LPS (50 ng/ml) for the indicated times, and iNOS expression was analyzed by Western blotting as indicated.

In mouse macrophages, the cellular response to LPS is transmitted from the plasma membrane to the cytoplasm through the Toll-like receptor 4/MD-2 complex in concert with CD14 and LPS-binding protein. In turn, many signal transduction pathways and transcription factors are activated [24]. Because the iNOS and TNF- α promoters contain main regulatory regions incorporating binding sites for NF- κ B and activator protein 1 (AP-1) [25, 26], we decided to study the implication of both flavonoids on these transcription factor signaling pathways in LPS-activated macrophages.

Although the JNK/stress-activated protein kinase (SAPK) pathway, which is implicated in the direct and indirect activation of AP-1, is activated preferentially by cellular stress signals such as irradiation, heat shock, osmotic stress and protein synthesis inhibitors [27], it is also activated by LPS (Fig. 4A) [19]. The LPS-induced activation of JNK was blocked by the pre-incubation of macrophages with the specific inhibitor SP600125 (25 μ M; Fig. 4A). However, neither quercetin (Fig. 4A) nor quercitrin (data not shown) were able to inhibit the c-Jun phosphorylation induced by LPS. These results suggest that JNK activation might not be implicated in the inhibition of TNF- α /IL-1 β and iNOS expression induced by quercetin in these cells.

In order to study the effect of quercetin/quercitrin on the NF- κ B pathway, we analyzed the I κ B- α phosphorylation by Western blotting (Fig. 4B). Our results show that quercetin, but not quercitrin, inhibited in a dose-dependent manner the phosphorylation of the I κ B- α protein induced by LPS treatment in macrophages (Fig. 4B), hence inhibiting the activation of the NF- κ B pathway.

So far, our results show that quercetin, but not quercitrin, exerts a potent *in vitro* anti-inflammatory effect which could be mediated through down-regulation of the NF- κ B pathway. Although *in vitro* experiments clearly showed that quercetin, but not quercitrin, had anti-inflammatory effects, previous studies of our group have shown that quercitrin [12], but not quercetin (unpublished results), is able to ameliorate the inflam-

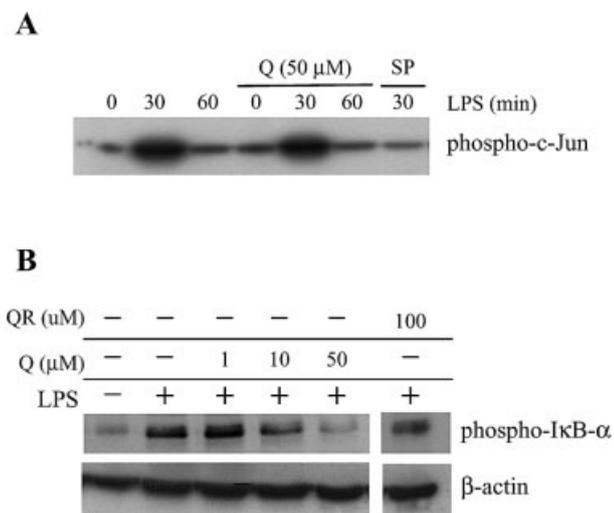


Fig. 4. Quercetin, but not quercitrin, inhibits LPS-induced I κ B- α phosphorylation but does not affect LPS-induced JNK activity. (A) Quiescent macrophages were incubated with quercetin (Q; 50 μ M) or SP600125 (SP; 25 μ M) for 1 h and then stimulated with LPS (50 ng/ml) for the indicated periods. JNK activation was measured by the *in vitro* phosphorylation of GST-c-Jun. (B) Quiescent macrophages were pretreated with quercetin (Q; 1, 10, 50 μ M) or quercitrin (QR; 100 μ M) for 1 h and then incubated with LPS (50 ng/ml) for 5 min. I κ B- α phosphorylation was analyzed by Western blotting. These experiments were performed three times with similar results.

matory process induced in the trinitrobenzene sulfonic acid (TNBS) model of rat colitis. Now, we extended our results obtained with these flavonoids in the experimental model of colitis after addition of DSS to drinking water, with special attention to the action exerted by the flavonoid treatment on colonic pro-inflammatory cytokine secretion, *i.e.* TNF- α and IL-1 β , and establishing its correlation with the induction of iNOS expression that takes place as a consequence of the inflammatory process induced by DSS.

The results obtained in the present study show that exposure to DSS in the drinking water induced a colonic inflammatory status with similar features to those described previously [28, 29], and it was evidenced by the time course increase in the disease activity index (DAI) values in comparison with non-colitic rats (Fig. 5). Oral treatment of colitic rats with quercitrin (1 mg/kg) ameliorated the inflammatory process as evidenced by a significant reduction in the DAI values when compared with untreated colitic rats (Fig. 5). On the contrary, quercetin treatment (1 mg/kg) of colitic rats was devoid of any beneficial effect on the colonic inflammatory status induced in rats by DSS (Fig. 5).

In addition, the inflammatory status of the intestine was also assessed biochemically by determining colonic myeloperoxidase (MPO) activity, an enzymatic marker of neutrophil infiltration. Colonic levels of MPO in colitic rats were increased in comparison with non-treated

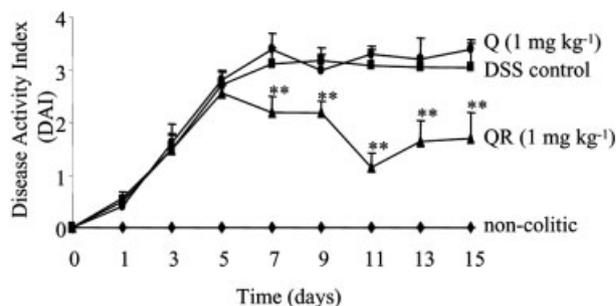


Fig. 5. Effects of quercitrin (QR) and quercetin (Q) treatment (1 mg/kg/day) on the time course changes in the DAI over the 15-day experimental period in DSS model of rat colitis. Treatment with quercitrin significantly attenuated the evolution of the colonic inflammatory process from day 7 up to the end of the study. ** p <0.01 vs. DSS control group.

colitic rats (131.0 ± 2.4 vs. 15.1 ± 1.4 U/g tissue; p <0.01). Quercitrin treatment of colitic rats significantly reduced colonic MPO activity (77.3 ± 6.1 U/g tissue; p <0.01 vs. non-treated colitic rats), whereas quercetin treatment did not affect MPO activity induced by DSS treatment (138.3 ± 4.8 U/g tissue; p >0.1 vs. non-treated colitic rats). These results confirm the intestinal anti-inflammatory effect of quercitrin evidenced in the course of the experiment by the DAI index evolution. The lack of beneficial effect by quercetin on colonic inflammation was also evidenced by other biochemical parameters assayed but due to simplification reasons not indicated in the following results.

When colon homogenates from colitic rats were used to evaluate the production of pro-inflammatory cytokines described in the *in vitro* experiments with BMDM, an increased production of both cytokines, TNF- α (Fig. 6A) and IL-1 β (Fig. 6B), was observed. The treatment with quercitrin almost completely inhibited the production of these cytokines without showing statistical differences in comparison with non-colitic group (Fig. 6A, B), thus confirming its intestinal anti-inflammatory effect in this model of rat colitis. We also measured the iNOS protein expression by Western blotting in these colonic segments. The intestinal anti-inflammatory effect exerted by quercitrin was associated with a significant inhibition of iNOS expression in the colon homogenates (Fig. 6C) when compared to DSS colitic animals without treatment.

Moreover, we studied whether the beneficial effect exerted by quercitrin was correlated with an inhibition of the NF- κ B or JNK pathways. The NF- κ B activity in the *in vivo* model of colitis was evaluated using an ELISA technique. Colon homogenates obtained from DSS colitic rats presented significant levels of activated NF- κ B molecules (expressed as relative luminescence units; 52.160 ± 5.911 in non-colitic rats vs. 85.128 ± 9.007 in non-treated colitic rats; p <0.05), which were down-

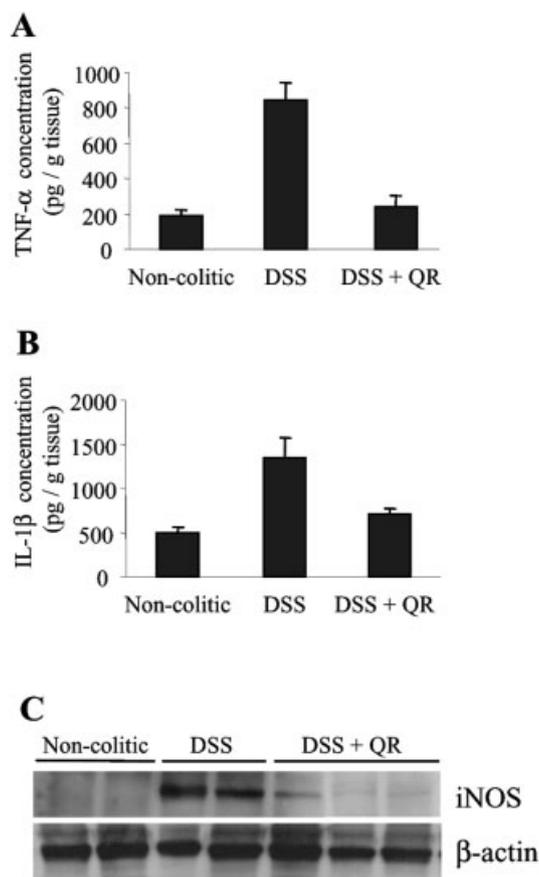


Fig. 6. Quercitrin treatment (1 mg/kg/day) inhibits cytokine and iNOS expression induced by DSS in rats. (A, B) Colon homogenates were used to analyze the expression of TNF- α (A) and IL-1 β (B) using an ELISA technique. Data are expressed as means \pm SEM, $n=8$. (C) Quercitrin (QR) inhibits iNOS expression induced by DSS in rats. Colonic iNOS expression was measured by Western blot.

regulated to normal values after quercitrin treatment of the colitic rats (57.554 ± 11.345). However, no significant effect was observed on JNK activation after quercitrin treatment (data not shown).

The paradoxical results obtained when compared *in vitro* and *in vivo* experiments with both flavonoids, quercetin and its glycoside quercitrin, *i.e.* the activity exerted by quercetin *in vitro* but not *in vivo*, as opposed for quercitrin, may be explained on the basis of the bioavailability of flavonoids. It is generally considered that aglycones, like quercetin, can be absorbed in the small intestine, preventing them from a local beneficial effect in the inflamed colon. On the contrary, flavonoid glycosides, like quercitrin, are not well absorbed in the upper segments of the gastrointestinal tract, thus reaching the colon, where they should be hydrolyzed by the intestinal microbiota, releasing the aglycone quercetin, responsible for the beneficial effects on colonic inflammation evidenced in the present study. For this reason we decided to check if this is really the case by performing fermentation studies with rat feces.

The results obtained in these experiments demonstrated that after inoculation with quercitrin, intestinal bacteria were able to hydrolyze quercitrin to quercetin (Fig. 7A, B). The involvement of bacterial components was further demonstrated by autoclaving the fecal sample before inoculation of quercitrin. In this situation, no quercetin production was detectable at any time point (data not shown). Moreover, the quercetin obtained by fecal fermentation was fully active. In this sense, 24-h-fermented quercitrin (dilution 1:50; the equivalent of approximately 50 μ M) was able to inhibit both TNF- α and IL-1 β secretion (Fig. 8A, B) and iNOS

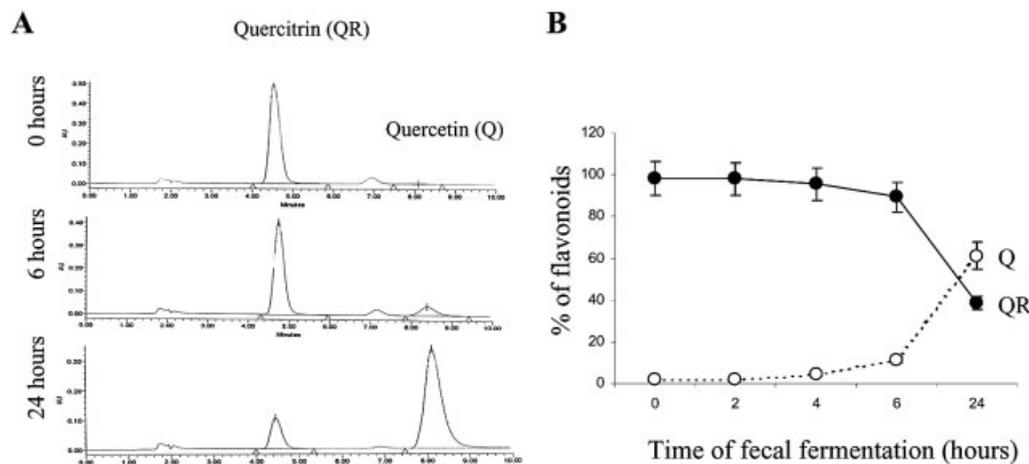


Fig. 7. Fecal microbiota is able to release quercetin (Q) from its glycoside quercitrin (QR). Rat fecal samples (50 mg/ml) were incubated in presence of 5 mg/ml quercitrin for the indicated times and analyzed by HPLC. HPLC histograms are shown in (A) and quantification of the area peaks is represented in (B). Pure quercetin and quercitrin solutions of known concentrations were used as standards. Values are represented as mean \pm SD of three independent experiments.

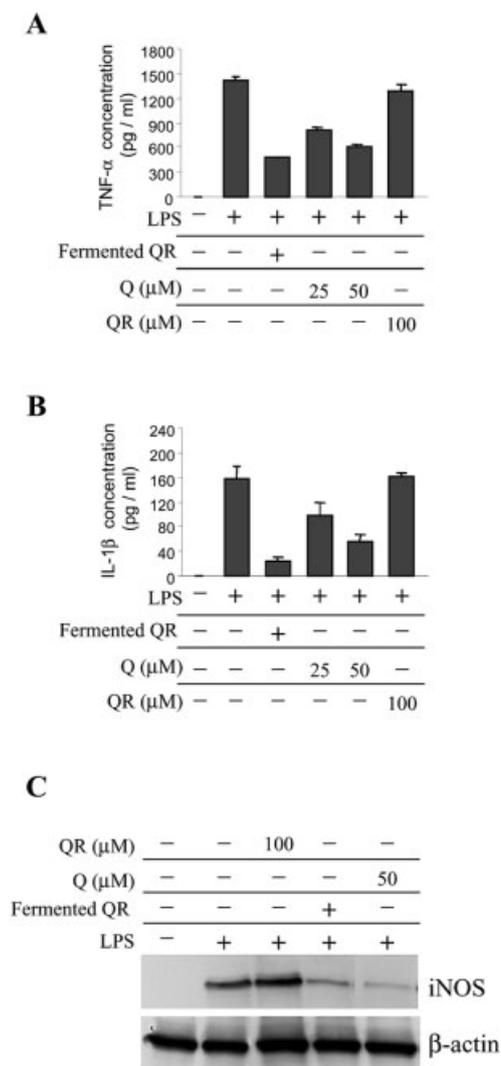


Fig. 8. Supernatants obtained from fermented quercitrin inhibit cytokine and iNOS expression induced by LPS in BMDM. (A, B) Cells (10^5) were incubated with supernatant from 24-h-fermented quercitrin (dilution 1:50), quercetin (Q; 25 and 50 μ M) and quercitrin (QR; 100 μ M) for 1 h and then stimulated with LPS (50 ng/ml) for 24 h. TNF- α (A) and IL-1 β (B) secretion was analyzed by ELISA. (C) iNOS expression induced by LPS is inhibited by fermented quercitrin. Macrophages were incubated as in (A, B) and then stimulated with LPS (50 ng/ml) for 10 h. The iNOS expression was measured by Western blot.

expression (Fig. 8C) produced by LPS stimulation in macrophages in a similar way than what was observed in the previous *in vitro* experiments with quercetin.

Discussion

Although the etiology of IBD is unknown, several evidences suggest that this intestinal condition is mediated by immunological alterations that lead, at least initially, to the activation of nonlymphoid cells such as macrophages and the subsequent release of pro-

inflammatory cytokines including TNF- α [13, 14, 18]. For this reason, actual therapeutic treatments of IBD include drugs, such as 5-aminosalicylic acid derivatives and systemic or local glucocorticoids, that exert beneficial effects through a combination of different mechanisms which result in the down-regulation of the exacerbated immune response that characterizes these pathologies. However, the characteristics of this inflammatory process imply the use of these drugs during prolonged time periods, and unfortunately, these drugs are not devoid of potentially serious side effects which limit their use [30]. For this reason, the development of new drug treatments that combine efficacy and safety is an important goal in IBD therapy. This could be the case for flavonoids, natural products that exert several biological activities, mainly related to their ability to inhibit enzymes and/or their antioxidant properties, and that have also been described to down-regulate the immune response [4]. These activities could support their consideration as valid drugs in the pharmacological treatment of IBD.

Quercetin is the most ubiquitous flavonoid in nature, including foods, which is usually present in glycosylated form such as quercitrin. For this reason, we analyzed the potency and efficacy of both quercetin and quercitrin as anti-inflammatory compounds. We decided to evaluate their *in vitro* effects in inhibiting macrophage functions, immune cells that are extensively involved in the initiation and progression of the inflammation process observed in IBD [13, 14]. Our results showed that quercetin, but not quercitrin, was able to inhibit both macrophage proliferation and activation, measured as [3 H]thymidine incorporation induced by M-CSF or iNOS and cytokine expression induced by LPS, in accordance with previous results reported by others [31]. These results would suggest that the use of the aglycone quercetin should be the best choice for the treatment of IBD. In contrast to what we could expect, we have not been able to demonstrate a therapeutic or preventive effect after oral administration of quercetin in two experimental models of rat colitis: TNBS model (unpublished results) and DSS model (present study). Meanwhile, previous studies have demonstrated the beneficial effects of two quercetin glycosides, rutin and quercitrin, in experimental models of rat colitis [11, 12], which have also been demonstrated in the present study for quercitrin.

The discrepancies between *in vivo* and *in vitro* anti-inflammatory effects of the aglycone or the glycoside may be explained when we consider that the glycoside quercitrin acts as a pro-drug which enables the transport and release of the aglycone quercetin in the colonic lumen exerting its local beneficial effect. In fact, it has been reported that most of the glycosides probably resist acid hydrolysis in the stomach and thus arrive intact in

the duodenum. Only aglycones, like quercetin, and some glucosides can be absorbed in the small intestine, whereas aglycones linked to a rhamnose moiety, like quercitrin, must reach the colon where they are hydrolyzed by rhamnosidases of the colonic microflora releasing the aglycone [32]. This is supported by the fermentation studies of quercitrin simulating the colonic environment that have been performed in the present study. Our results demonstrated that fecal microbiota is able to hydrolyze glycosides and also that the resultant aglycones are fully active and able to inhibit macrophage activation in a similar way as pure quercetin.

The second aim of this work was to explore the mechanism of action of quercetin related to its anti-inflammatory properties. In this sense, although the antioxidant properties of quercetin could exert a beneficial effect in inflammation as has previously been described [33, 34], it is probable that additional mechanisms are also involved. Both *in vitro* and *in vivo* studies suggest that quercetin ameliorates inflammation by inhibition of either the secretion of inflammatory mediators such as nitrogen reactive species produced after induction of iNOS expression or the expression and subsequent release of cytokines such as TNF- α and IL-1 β . When we assayed the effects of quercetin on two transcription factor pathways involved in the gene transcription of both iNOS and TNF- α induced by LPS, our results showed that the anti-inflammatory effect of flavonoids may be associated with down-regulation in NF- κ B activity, without significantly affecting the JNK signaling pathway.

It is well documented that the NF- κ B pathway can be inhibited by a variety of structurally diverse antioxidants, such as polyphenols [33, 35]. The molecular mechanisms involved in the suppressive effects of flavonoids on NF- κ B are currently unknown, but several possibilities have been postulated. Flavonoids may inhibit NF- κ B by acting as antioxidants, since NF- κ B is a redox-sensitive transcription factor and activated by oxidant stress in the inflamed intestinal mucosa [36]. However, again this is pointed out in this work due to the fact that although quercitrin retained its antioxidant potential, it was unable to modulate NF- κ B activation in the *in vitro* experiments. As a consequence, other mechanisms such as direct inhibition of intracellular signal transduction pathway leading to NF- κ B activation may be exerted by these flavonoids. Future studies from our group are directed to elucidate this possibility.

The inability of quercetin to ameliorate colon inflammation in animal models could be related to the fact that quercetin was rapidly absorbed in the upper intestinal portion and disseminated through the blood stream without being able to reach pharmacological effective concentrations at the required site, *i.e.* the inflamed colon. Probably, higher concentrations should

be needed in order to reach anti-inflammatory effects in the colon, such as that described with morin [37]. In contrast, the glycosylated form of quercetin is protected from absorption and reaches the last portion of the intestine intact and in high amounts. There, in the colon, fecal microbiota will be able to hydrolyze it, releasing functional quercetin in sufficient amounts in order to perform the local anti-inflammatory effects needed for the IBD treatment. New therapeutic strategies such as the use of glycoside conjugates in conjunction with substrates (prebiotics) or microorganisms (probiotics) that promote their colonic hydrolysis and release could be of potential interest in IBD and are currently under exploration in our group.

Materials and methods

Reagents

Quercitrin was purchased from Extrasynthèse (Genay, France). DSS was provided by ICN Biomedicals (Costa Mesa, CA). All other reagents, unless otherwise stated, were obtained from Sigma (Madrid, Spain).

Animals

Female Wistar rats (170–190 g) and male BALB/c mice were obtained from Granada University breeding colony and housed under temperature- (22°C) and light-controlled (12 h) conditions. This study was carried out in accordance with the Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes of the European Union (86/609/EEC) in compliance with the Helsinki declaration.

Macrophage culture and proliferation assay

BMDM were obtained as previously described [19]. Cells were cultured in Dulbecco's modified Eagle's medium which contained 20% FCS and 30% L cell-conditioned medium as source of M-CSF. After 6 days of culture, macrophages were deprived of L cell-conditioned medium for 16–18 h. Then, the cells were incubated in the presence or absence of the indicated flavonoids and proliferation was measured by [³H]thymidine incorporation as previously described [21]. Each point was performed in triplicate and results are expressed as the mean \pm SD.

Induction of colitis and assessment of colonic damage

After a 7-day acclimation period, 32 rats were weighed and randomly distributed to the different experimental groups ($n=8$ per group). Rats were rendered colitic by replacing normal drinking water with distilled water containing 5% DSS (w/v, prepared daily, molecular weight 36,000–50,000) for the first 5 days, reduced down to 2% (w/v) for the following 10 days [22]. Four experimental groups of rats were included: non-colitic, control colitic and two flavonoid-treated groups,

with quercitrin or quercetin. Flavonoid treatment (at the dose of 1 mg/kg/day) started when the concentration of DSS was reduced, dissolving the flavonoid in distilled water and administering it orally by means of an esophageal catheter. Controls received equivalent volumes of water. Food and water consumption was also recorded daily throughout the study. Animals were sacrificed after 15 days from the beginning of the experiment.

Rat body weight, the presence of gross blood in the feces, and stool consistency were recorded daily throughout the experiments. A score was assigned to each of these parameters according to the criteria proposed by Cooper et al. [38], and used to calculate an average daily DAI for each animal. Once rats were killed by cervical dislocation, their colons were immediately removed and rinsed with ice-cold PBS and processed for colonic cytokine production, iNOS expression and MPO activity. MPO activity was measured according to the technique described by Krawisz et al. [39] and the results were expressed as MPO units per gram of wet tissue; 1 unit of MPO activity was defined as that degrading 1 mmol/min of hydrogen peroxide at 25°C.

Protein extraction, Western blot analysis and JNK activity assay

Colonic samples obtained from rats were homogenized in PBS (1:3 w/v) with 0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100 and protease inhibitors. For *in vitro* samples, macrophages were washed with PBS and lysed in cold lysis buffer (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 protease inhibitors). JNK activity assays were performed as described elsewhere [19]. Western blots were performed as described [19] with minor modifications. Cell lysates (100 μ g for iNOS, TNF- α and IL-1 β detection and 50 μ g for phospho-I κ B- α detection) were loaded. The dilutions of antibodies used were: 1:2,000 iNOS (Transduction Laboratories, BD Biosciences, Madrid, Spain), 1:2,000 phospho-I κ B- α (Cell Signaling Technology, Beverly, MA), 1:1,000 TNF- α (Bender MedSystems, Wien, Austria) and 1:1,000 IL-1 β (Santa Cruz Biotechnology, Santa Cruz, CA). A primary antibody against β -actin was used as loading control and purchased from Sigma. Peroxidase-conjugated anti-mouse IgG was used as secondary antibody. All antibody incubations were performed at 4°C overnight.

Determination of pro-inflammatory mediators

The secretion of TNF- α and IL-1 β in macrophage cultures was measured using a commercial mouse TNF- α or IL-1 β ELISA kit (Cytosets™, Biosource International, Nivelles, Belgium). Cells (10⁵) were cultured in 24-well plates in the presence or absence of quercetin (1, 10, 50 μ M) and quercitrin (100 μ M) during 1 h and then stimulated with LPS (100 ng/ml). Supernatant samples were obtained 16 h later and frozen until subjected to ELISA analysis following the manufacturer's protocol. The TNF- α and IL-1 β production in colon homogenates was quantified by ELISA (Amersham, Madrid, Spain), and expressed as pg/g tissue.

Micro-well colorimetric NF- κ B assay

Colonic NF- κ B activation was determined by an ELISA kit (TransAM™ method; Carlsbad, CA). Colon homogenates were suspended in lysis buffer (20 mM Hepes pH 7.5, 0.35 M NaCl, 20% glycerol, 1% Nonidet-P40, 1 mM MgCl₂·6H₂O, 0.5 mM EDTA, 0.1 mM EGTA) containing protease and phosphatase inhibitors (aprotinin, leupeptin, 1,10-phenanthroline, PMSF, ioadacetamide and sodium orthovanadate). After 10 min on ice, the lysate was centrifuged for 20 min at 12,000 \times g, with the supernatants containing the total protein extract being stored at -80°C until subjected to analysis. Each protein extract (10 μ g) was incubated in a 96-well plate coated with immobilized oligonucleotide containing the NF- κ B consensus site (5'-GGGACTTCC-3'). The active form of NF- κ B contained in cell extracts specifically binds to this oligonucleotide and can be detected by ELISA. The ELISA kit contains a positive control from Jurkat cells stimulated with TPA and calcium ionophore.

Fecal fermentation of quercitrin and HPLC analysis

Fecal fermentation of quercetin was performed by diluting 100 mg of rat feces in 1 ml of peptone water (Oxoid, Basingstoke, UK) added with quercitrin (10 mM) and incubated anaerobically at 37°C for the indicated times. After that, samples were 0.22- μ m filtered and used for the *in vitro* assays or extracted with 1 volume of methanol, vortexed for 1 min, centrifuged at 5,000 \times g for 5 min, and analyzed by HPLC for the contents of quercetin and quercitrin.

HPLC analysis was performed by a Waters 2695 coupled to a photodiode Array detector. The analysis conditions were: column Hypersil BDS-C18 100 \times 4.0 mm I.D., 3 μ m particle size (Agilent Technologies); column temperature 25°C; mobile phase, phase A methanol and phase B acetic acid 2% (v/v); flow rate 0.6 ml/min; injection volume 10 μ l. The mobile phase gradient profile was as follows: t=0 min, A=60%; t=10 min, A=80%; t=11 min, A=60%; t=15 min, A= 60%. Analytes were detected at 360 nm.

Statistical analysis

All results are expressed as means \pm SEM, except for TNF- α and IL-1 β concentration in macrophage culture supernatants, which were expressed as means \pm SD. Differences among means were tested for statistical significance using one-way analysis of variance (ANOVA) and *post-hoc* least-significance tests. Statistical significance was set at $p < 0.05$.

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