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Rutin has intestinal antiinflammatory effects in the CD4+ CD62L+ T cell transfer model of colitis

Cristina Mascaraque[a], Carlos Aranda[b], Borja Ocón[a], María Jesús Monte[c], María Dolores Suárez[b], Antonio Zarzuelo[a], José Juan García Marín[c], Olga Martínez-Augustin[b], Fermín Sánchez de Medina[a,*]

[a] Department of Pharmacology, CIBERehd, School of Pharmacy, University of Granada, Campus de Cartuja s/n, 18071 Granada, Spain
[b] Department of Biochemistry and Molecular Biology II, CIBERehd, School of Pharmacy, University of Granada, Campus de Cartuja s/n, 18071 Granada, Spain
[c] Department of Physiology and Pharmacology, HEVEFARM, IBSAL, CIBERehd, University of Salamanca, Spain

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ABSTRACT

Rutin, one of the most abundant flavonoids in nature, has been shown to exert intestinal antiinflammatory effects in experimental models of colitis. Our aim was to study the antiinflamatory effect of rutin in the CD4+ CD62L+ T cell transfer model of colitis, one of the closest to the human disease. Colitis was induced by transfer of CD4+ CD62L+ T cells to Rag1$^{-/-}$ mice. Rutin was administered by gavage as a postreatment. Treatment with rutin improved colitis at the dose of 57 mg/kg/day, while no effect was noted with 28.5 mg/kg/day. Therapeutic benefit was evidenced by a reduced disease activity index, weight loss and damage score, plus a 36% lower colonic myeloperoxidase and a 54% lower alkaline phosphatase activity. In addition, a decreased secretion of proinflammatory cytokines (IFNγ and TNFα) by mesenteric lymph node cells was observed ex vivo. The colonic expression of proinflammatory genes, including IFNγ, TNFα, CXCL1, S100A8 and IL-1β, was significantly reduced by more than 80% with rutin as assessed by RT-qPCR. Flavonoid treated mice exhibited decreased activation of splenic CD4+ cells (STAT4 phosphorylation and IFNγ expression) and reduced plasma cytokine levels. This effect was also apparent in mucosal lymphocytes based on reduced STAT4 phosphorylation. The protective effect was comparable to that of 3 mg/kg/day budesonide. Rutin had no effect on splenocytes or murine T cells in vitro, while its aglycone, quercetin, exhibited a concentration dependent inhibition of proinflammatory cytokines, including IFNγ. Rutin but not quercetin showed vectorial basolateral to apical transport in IEC18 cells, associated with reduced biotransformation. We conclude that rutin exerts intestinal antiinflammatory activity in chronic, T lymphocyte dependent colitis via quercetin release and actions involving mucosal and lymph node T cells. Our results suggest that rutin may be useful in the management of inflammatory bowel disease in appropriate dosage conditions.

Introduction

Inflammatory Bowel Disease (IBD), comprising mainly Crohn's disease and ulcerative colitis, is a chronic condition characterized by persistent and recurrent intestinal inflammation, manifested clinically with abdominal pain, diarrhea, malabsorption, general malaise, etc. While there are important immunological and histopathological differences between both entities, IBD as a whole is generally considered the product of an infortunate interplay of genetic and environmental factors resulting in an exacerbated immune response toward luminal antigens [1]. IBD patients are normally managed successfully with drug therapy but this is unsatisfactory because of important side effects, the frequent need for surgery and therapy-resistant flares of the disease. Thus there is a

Abbreviations: AP, alkaline phosphatase; ConA, concanavalin A; COX2, cyclooxygenase 2; CXCL1, chemokine (C-X-C motif) ligand 1; DAI, disease activity index; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; IκB-α, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; MLNC, mesenteric lymph node cells; MMP3, matrix metalloproteinase-3; MPO, myeloperoxidase; Rag1, recombination activating gene 1; REG3γ, regenerating islet-derived 3 γ; STAT4, signal transducer and activator of transcription; TNBS, trinitrobenzenesulfonic acid; TNFα, tumor necrosis factor alpha; Treg, regulatory T cells.

* Corresponding author. Tel.: +34 958 241747.
E-mail addresses: cristimascaraque@gmail.com (C. Mascaraque), cjarandaclemente@gmail.com (C. Aranda), boryus13@hotmail.com (B. Ocón), mjmonte@usal.es (M.J. Monte), msuarez@ugr.es (M.D. Suárez), zarzuelo@ugr.es (A. Zarzuelo), jjgmarin@usal.es (J.J.G. Marín), omartine@ugr.es (O. Martínez-Augustin), fsanchez@ugr.es (F.S. de Medina).

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well recognized demand for new and improved treatments for IBD [2].

Flavonoids are a family of polyphenolic compounds which are widespread in nature (vegetables) and that are consumed as part of the human diet in significant amounts. Flavonoids are bioactive compounds that display a number of biological activities, which have been reviewed [3,4]. These include antioxidant/antiradicalary properties and a number of immunomodulatory activities. Our group and several others have studied the intestinal antiinflammatory effects of a number of flavonoids, including rutin [5,6], based on the regular exposure of humans to oral flavonoids in the diet and their biological properties. Thus rutin (α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranose) has been shown to exert beneficial effects in acetic acid [7], trinitrobenzenesulfonic acid (TNBS) [8] and dextran sulfate sodium (DSS) rat colitis [9,10]. A closely related flavonoid glycoside, quercitrin (quercetin-3-rhamnose), has also been reported to have inflammatory effects in preclinical IBD models, namely rat TNBS colitis [11,12].

Preclinical models of IBD are widely used to study pathophysiological aspects of the condition and to test new experimental treatments, including flavonoids. By far animal studies have employed chemically induced models of colitis, specially the TNBS and DSS models, due to their simplicity and reproducibility, and the fact that they feature some of the characteristics of the human condition. It should be noted that there is no ideal model of IBD, and in this regard the TNBS and DSS models are useful. However, they also present several disadvantages because they are not strictly chronic (i.e. they heal with time) and they do not depend on lymphocytes to develop as in the human disease, since they can be induced with very similar features in rodents devoid of lymphocytes [13,14] or T cells [15]. Some authors have advocated the use of the T cell transfer model of colitis to achieve a better prediction of human bioactivity [16]. In order to fully validate the possible use of rutin in IBD, it is important to demonstrate its bioactivity in such a model. This point may be particularly relevant for rutin since one study that tested the effect of dietary flavonoid supplementation in MDR1A knockout colitic mice found no evidence of therapeutic activity [17]. Hence we set out to verify the antiinflamatory effect of rutin in the CD4+ CD62L+ T cell transfer model of colitis. We additionally generated in vitro data to further characterize the mechanism of action of the flavonoid.

Materials and methods

Reagents

Except where indicated, all reagents and primers were obtained from Sigma (Barcelona, Spain). Reverse transcription was achieved with the iScript™ cDNA Synthesis Kit and iQ™ Sybr® Green Supermix was used for amplification (Biorad, Alcobendas, Madrid, Spain). All the primary antibodies used in the magnetic separation were purchased from BD Pharmingen™ (Madrid, Spain); MACS columns, anti-biotin and CD62L microbeads were provided by MACS Miltenyi Biotec (Cologne, Germany). Mouse ELISA kits (IL-6, TNFα, IL-10, IFNγ and IL-17) were obtained from eBioscience (San Diego, CA, USA) and rat ELISA kits IL-2 (Biosource® Europe, Nivelles, Belgium), IFNγ and TNFα (BD Biosciences®, Erembodegem, Belgium). Budesonide was purchased from Molekula Ltd. (Gillingham, UK).

Animals

All animal procedures in this study were 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 were approved by the Animal Welfare Committee of the University of Granada (registry number 710).

Thirty-two female C57BL/6J wild type (cell donors and noncolitic controls) and 59 $Rag1^{-/-}$ mice (cell receptors) were obtained from Jackson Laboratory (CA, USA). In addition five Wistar rats (female, 200 g) were used to obtain isolated T cells. Animals were maintained at the Unit of animal research (Biomedical Research Center, University of Granada, Granada, Spain) in air conditioned animal quarters with a 12 h light–dark cycle. Animals were housed in specific pathogen free conditions, in individual ventilated cages with an air insufflation and exhalation system with dual filter (prefilter and HEPA filter), and were given free access to autoclaved tap water and food (Harlan-Teklad 2014, Harlan Ibérica, Barcelona, Spain).

Induction of transfer colitis and experimental design

Female C57BL/6J mice were sacrificed at 16 weeks of age by cervical dislocation and the spleen was extracted aseptically. Cell suspensions were obtained by disrupting the tissues between dissecting forceps in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%), 2 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 2.5 mg/mL amphotericin B. After centrifuging, cells were cleared of erythrocytes by suspension on hypotonic lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM $Na_2EDTA{\cdot}2H_2O$, pH 7.3) for 30 min on ice. Cells were filtered using a 70 μm filter (cell strainer BD Falcon™, Ref. 352350) to obtain a mononuclear suspension. Mononuclear cells were washed and resuspended in MACS buffer (0.5% bovine serum albumin and 2 mM EDTA in PBS, pH 7.2). CD4+ CD62L+ T cell isolation from spleen cells was performed using the CD4+ CD62L+ T Cell Isolation Kit II (Miltenyi Biotec, Cologne, Germany). First, non-CD4+ T cells were indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies and anti-biotin microbeads. The labeled cells were subsequently depleted by separation over a MACS column. In the second step, CD4+ CD62L+ T cells were directly labeled with CD62L (L-selectin) microbeads and isolated by positive selection from the pre-enriched CD4+ T cell fraction. The CD4+ CD62L+ T cells were eluted in 100 μL of sterile PBS and administered intraperitoneally into C57BL/6J $Rag1^{-/-}$ mice (1×10^6 CD4+ CD62L+ T cells per mouse). The non-colitic control group ($Rag1^{-/-}$ background) was administered sterile PBS (without CD4+ CD62L+ T cells). An additional group of mice that were injected with unfractionated splenocytes showed little or no sign of inflammation after up to 12 weeks (data not shown).

The status of the animals was monitored by general examination and specifically controlling body weight evolution, fecal blood and diarrhea. Treatment was started when colitis was established taking into account these parameters (8 weeks after transfer). Only mice with established disease were used. After the beginning of the treatment animal status was assessed every other day as a disease activity index (DAI), which was the sum of diarrhea (0–3), blood in feces (0–2) and body weight evolution (0–1).

Colitic mice were randomly assigned to two different groups, i.e. the control ($n = 10$) and the rutin group ($n = 10$), which received vehicle (water) or rutin by gavage (0.1 mL volume), respectively. In addition there was a non-colitic control group (no transfer group, $n = 6$). Two doses of rutin were separately administered by gavage, 28.5 and 57 mg/kg/day (i.e. two different experiments). A period of treatment of 12 days was preestablished in order to assess a stable effect of the flavonoid, as judged from body weight evolution and the DAI, taking advantage of the chronic nature of the model. However, with the lower dose of rutin treatment was continued longer (22 days) to confirm the lack of effect (see below). Animals were sacrificed by cervical dislocation under isoflurane anesthesia.