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Molecular mechanisms of gastrointestinal protection by quercetin against indomethacin-induced damage: role of NF-κB and Nrf2

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Abstract

The aim of this study was to determine the gastrointestinal protection by quercetin against indomethacin-induced oxidative stress and inflammation, with specific interest in studying the underlying molecular mechanisms. We hypothesized that the quercetin-protective effect relies on its antioxidant and antiinflammatory properties. Rats were pretreated with quercetin (50- or 100-mg/kg, ig single dose), 30 min before INDO administration (40-mg/kg ig single dose). Caco-2 cells were treated with INDO (250 and 500 μM) in the absence or presence of quercetin (10 μg/ml). Quercetin prevented the decrease in nuclear translocation of Nrf2, a key regulator of the antioxidant response, and the increase in reactive oxygen species levels induced by INDO by inhibiting the enhancement of NADPH oxidase and xanthine oxidase activities as well as the reduction in superoxide dismutase and glutathione peroxidase activities in gastric and ileal tissues. Quercetin also prevented INDO-induced ICAM-1 and P-selectin expressions and the increase of myeloperoxidase activity in gastric and ileal tissues and NF-κB activation and IL-8 production in Caco-2 cells. Quercetin did not affect the inhibition of TNFα-mediated production of prostaglandin E_2 induced by INDO in Caco-2 cells. The protective effects of quercetin observed in the gastric and ileal mucosa of rats as well as in Caco-2 cells relied on the ability of this flavonol to prevent NF-κB activation and increase Nrf2 translocation. This study supports the concept that quercetin may be useful in the prevention and/or treatment of nonsteroidal antiinflammatory drug-associated side effects, without interfering with their therapeutic efficacy.

Keywords: Quercetin; Indomethacin; NF-kB; Nrf2; Oxidative stress; Inflammation

1. Introduction

Nonsteroidal antiinflammatory drugs (NSAIDs) are widely used for their antipyretic, analgesic and antiinflammatory properties [1]. However, their administration is frequently associated with adverse effects that mainly affect the gastrointestinal (GI) mucosa. Indomethacin (INDO) is one of the most damaging NSAIDs for the GI mucosa; thus, it is frequently used as a paradigm drug to study the adverse effects associated with NSAID use and to evaluate the potential protective effect of bioactive compounds. We recently reported on Caco-2 cells and animal models of GI damage that the mechanisms associated with the cytotoxic events induced by INDO in the GI tract are linked to mitochondrial dysfunction, oxidative stress and apoptosis [2–7]. Specifically in terms of oxidative stress, we showed that INDO decreased the reduced (GSH)/oxidized glutathione (GSSG) ratio and increased the dichlorofluorescein oxidation, superoxide radical production, xanthine oxidase (XO) activity and lipid peroxidation in Caco-2 cells [2–4]. In addition, we observed that oral administration of INDO to rats induced GI mucosal damage and that these effects were associated with an increase in lipid peroxidation and a decrease in the GSH/GSSG ratio [7]. The molecular mechanisms related to redox homeostasis have not been characterized in these models of GI damage.

On the other hand, the proinflammatory effects of INDO have been linked to activation of nuclear transcription factor kappa B (NF-κB), the induction of migration/infiltration of the mucosa by polymorphonuclear leukocytes and the increased expression of proinflammatory cytokines and adhesion molecules like ICAM-1 [8,9]. These proinflammatory events are associated with a prooxidant effect, such as increasing NADPH oxidase expression and decreasing catalase activity [10]. Polymorphonuclear migration during an inflammatory response is mediated through interactions between adhesion

Abbreviations: EMSA, electrophoretic mobility shift assay; GCLC, glutamate-cysteine ligase catalytic subunit; GI, gastrointestinal; GSHpx, glutathione peroxidase; ICAM-1, intercellular adhesion molecule-1; IkBα, inhibitory subunit of kappa B; INDO, indomethacin; MAPK, mitogen activated protein kinase; MPO, myeloperoxidase; NF-κB, nuclear transcription factor kappa B; Nrf2, nuclear factor (erythroid-derived 2)-like 2; NSAID, nonsteroidal antiinflammatory drug; NQO1, NAD(P)H dehydrogenase [quinone] 1; $O_2^{\cdot -}$, superoxide radicals; QUE, quercetin; RFU, relative fluorescence unit; RLU, relative luminescence unit; ROS, reactive oxygen species; SOD, superoxide dismutase; TNFα, tumor necrosis factor alpha; XO, xanthine oxidase.

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molecules present in the membrane of endothelial cells and neutrophils. P-selectin mediates the rolling or slowing of neutrophils, whereas intercellular adhesion molecule-1 (ICAM-1) contributes to the firm adhesion and emigration of polymorphonuclear leukocytes [11]. Polymorphonuclear leukocyte migration, one of the major factors causing a predisposition to NSAID-induced gastric and intestinal lesions, consists of several steps, including interaction with P-selectin from platelets and endothelial cells [12].

Various synthetic antiulcer drugs are currently available, and some, such as prostaglandin analogs and proton pump inhibitors, are employed to manage NSAID-induced gastric ulcers [13]. However, these agents are believed to improve the clinical outcome without directly attacking the etiology of mucosal damage.

Quercetin (QUE) is a ubiquitous polyphenol present in fruits and vegetables. It represents one of the most abundant flavonoids in the western diet, and according to the US Department of Health and Human Services, the average human daily intake of QUE is 25 mg [14], data which are also supported by studies analyzing French and Dutch diets [15,16]. High amounts of QUE may be found in onions (284- to 486-mg/kg fresh edible part) and apples (21–72 mg/kg according the variety) [17]. Due to its low level of intestinal absorption, this flavonoid is accumulated in the GI tract, making this its main site of action [18]. QUE has been shown to exert its antioxidant and antiinflammatory effects by activating nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and inhibiting NF-κB pathways [19–21]. Nrf2 plays a central role in the redox homeostatic gene regulatory network by inducing the expression of phase II and antioxidant enzymes [22,23]. We have reported that a polyphenol-rich extract reduced the oxidative stress, mitochondrial dysfunction and cell death induced by INDO in *in vitro* and *in vivo* models of GI damage [2,3,7]. QUE has been reported to prevent INDO-induced mitochondrial dysfunction through the antioxidant mechanism in Caco-2 cells [4,6]. Although we have made advances in studying the oxidative and inflammatory damage induced by INDO and the protective effect of polyphenolic compounds, we have not yet characterized the underlying molecular mechanisms of GI protection induced by QUE.

The aim of this study was to determine the GI protection by QUE against INDO-induced oxidative stress and inflammation, with specific interest in studying the underlying molecular mechanisms. We hypothesized that the damage would be attenuated through the induction of antioxidant and antiinflammatory mechanisms. For this purpose, we used an *in vivo* and *in vitro* model of GI damage.

2. Material and methods

2.1. Animals

The study protocol was approved by the Animal Ethics Committee of the University of Chile, Faculty of Medicine; all the procedures were performed in compliance with the Guidelines for Care and Use of Laboratory Animals at the University of Chile. Thirty male Sprague Dawley rats (180–220 g, 7–8 weeks old) purchased from the Pontifical Catholic University, Santiago, Chile, were housed with a 12-h light/dark schedule at room temperature and fed standard rodent chow with *ad libitum* access to water. The animals were fasted for 15 h before the experiments, with water *ad libitum*.

2.2. Experimental design

The 30 rats were randomized into five groups. Group 1 (control): a single oral administration of vehicle (through gavage) consisting of 5% $NaHCO_3$, pH 7.0. Group 2 (QUE 100 mg/kg): a single dose of the flavonoid was administered orally at a dosage of 100-mg/kg body weight (bw), dissolved in 5% $NaHCO_3$, pH 7.0. Group 3 (INDO 40 mg/kg): a single dose of INDO was administered orally at a dosage of 40-mg/kg bw, dissolved in 5% $NaHCO_3$, pH 7.0. Group 4 (QUE 50 mg/kg + INDO 40 mg/kg): a single dose of 50-mg/kg bw of QUE was administered orally 30 min before a single dose of 40-mg/kg bw of INDO. Group 5 (QUE 100 mg/kg + INDO 40 mg/kg): a single dose of 100-mg/kg bw of QUE was administered orally 30 min before a single dose of 40-mg/kg bw of INDO. Four hours after INDO administration, the animals were sacrificed by decapitation after ketamine:xylazine anesthesia (100 mg/kg:10 mg/kg, ip), and stomach and ileum were removed immediately (Fig. 1).

2.3. Tissue preparation

The stomach was opened along its greater curvature and the ileum along its antimesenteric border; the tissues were washed in saline solution at 4°C, and segments from both organs were fixed in 4% paraformaldehyde for subsequent immunofluorescence analysis. The intestinal mucosa was scraped with a glass slide and stored at −80°C for further analysis as well as the remaining gastric tissue. For biochemical analysis, the gastric tissue was homogenized with an Ultra-Turrax (IKA T18 basic) and the ileal mucosa with a Teflon Dounce homogenizer (5 strokes). The homogenization process was carried out under a protein inhibitor cocktail (4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, bestatin, leupeptin and aprotinin; Sigma MO, USA). Myeloperoxidase (MPO), NADPH oxidase, SOD, glutathione peroxidase (GSHpx) activities, superoxide production and Nrf2 expression were quantified in the samples.

2.4. Cell culture conditions

The human intestinal epithelial cell line Caco-2 was cultured in DMEM-F12 with 10% fetal bovine serum added at 37°C (5% CO_2/95% air). Caco-2 cells were trypsinized when they reached 90% confluence, and differentiated cells were used for the experiments.

2.5. Immunofluorescence assays

The presence of ICAM-1 and P-selectin was detected in gastric and ileal tissues by immunofluorescence. Fixed tissue samples were embedded in paraffin, and two serial cross-sections (10 μm) from each sample were prepared and collected on poly-L-lysine coated glass slides. A total of 60 stomach samples and 60 ileum samples were analyzed. After deparaffinization and rehydration, tissues were subjected to antigen retrieval through incubation with proteinase K and serum blocking solution to prevent nonspecific binding. After this, sections were incubated overnight at 4°C with goat polyclonal anti-ICAM-1 antibody (Santa Cruz Biotechnology, CA, USA) diluted to 1:100 and goat polyclonal anti-P-selectin (Santa Cruz Biotechnology, CA, USA) diluted to 1:100. All sections were washed, stained with 0.25 μg/ml of DAPI and subsequently incubated with FITC-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature in the dark. After washing, coverslips were mounted with fluorescence mounting medium (Dako), and the immune reactivity was assessed by fluorescent microscopy (Nikon Eclipse E 400). The mean fluorescence intensity was obtained by using ImageJ (NIH Image, http://www.scioncorp.com, Scion Corporation, USA), taking into account the area of the tissue under observation according to the magnification. At least six different areas were counted per section, three sections were included per sample.

2.6. MPO activity

Neutrophil infiltration was assessed through the determination of MPO activity. The mucosa was homogenized in 50-mM PBS, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide (HETAB) and 10 mM EDTA. An aliquot of homogenate was added to a solution containing 80-mM PBS, pH 5.4, 0.5% HETAB and 1.6-mM 3,3',5,5'-tetramethylbenzidine, and the reaction was started by adding 0.3-mM hydrogen peroxide. Optical density was measured at 655 nm. One unit (U) of MPO activity was defined as the amount of enzyme that produced a change in absorbance of 1.0 unit/min at 37°C [7].

2.7. NF-κB activation

Caco-2 Cells were plated in 75 cm^2 flask plates at a density of 2×10^7 cells. After 24 h, cells were incubated for 4 h with TNFα, as a positive control to induce NF-κB nuclear translocation [24], and/or with INDO and QUE. NF-κB activation was assessed by the electrophoretic mobility shift assay (EMSA). EMSA remains a powerful experimental tool for detecting the presence of NF-κB bonded to DNA [25]. Nuclear protein extracts from the samples were prepared as previously described [26]. Nuclear extract (8 μg) was mixed with double-stranded NF-κB oligonucleotide 5-GATCTCAGAGGGGACTTTCC GAG-3 (GrupoBios SA, Chile) labeled with α-$^{32-}$P-dCTP using the Klenow DNA Polymerase Fragment I (Invitrogen Corp., Carlsbad, CA, USA) [27]. To confirm the specificity for NF-κB, a 100-fold excess of unlabeled NF-κB oligonucleotide was added to the reaction mixture as a competitor. Samples were loaded in nondenaturing conditions. NF-κB bands were detected by autoradiography and quantified by densitometry using ImageJ.

2.8. IL-8 quantitation

Caco-2 cells were plated in 24-well plates at a density of 1×10^5 cells/well. After 24 h, cells were incubated for 4 h with TNFα as a positive control of induction of IL-8 secretion [28], or with INDO (250 and 500 μM) alone or in the presence of QUE (10 μg/ml). Cells were also incubated for 4 h with TNFα and INDO to determine the eventual antiinflammatory effect of the NSAID. This experiment was also conducted in the presence of QUE to evaluate its ability to prevent the IL-8 secretion induced by the positive control (data not shown) as well as by INDO. The media from each well were collected and analyzed for IL-8 secretion using a Human IL-8 Ultrasensitive ELISA Kit (Invitrogen, CA, USA).