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Research Paper

Metabolic and Pharmacological Properties of Rutin, a Dietary Quercetin Glycoside, for Treatment of Inflammatory Bowel Disease

Heejung Kim,[1] Hyesik Kong,[2] Boim Choi,[3] Youngwook Yang,[3] Youngmi Kim,[3] Mi Jung Lim,[4] Len Neckers,[2] and Yunjin Jung[1,5]

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Purpose. Orally administered rutin reportedly ameliorates 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis of rats. We investigated the metabolic and pharmacological properties of rutin underlying the rutin-mediated amelioration of the rat colitis.

Methods. Apparent partition coefficients of rutin and its aglycone quercetin were compared. The biochemical/chemical stability of rutin was examined in the contents of various segments of gastrointestinal tracts of rats. Inflammatory indices were determined in the colitis rats after oral administration of rutin or rectal administration of quercetin. In human colon epithelial cells, the effect of quercetin on tumor necrosis factor-alpha (TNF-α)-induced nuclear factor kappa B (NFκB) activation was examined.

Results. The sugar residue in rutin greatly lowered the apparent partition coefficient and was rapidly deglycosylated to liberate quercetin in the cecal contents, whereas it was stable in the contents of the upper intestine. Not only oral administration of rutin but also rectal administration of quercetin remarkably ameliorated TNBS-induced colitis rats, indicating that quercetin liberated from rutin is therapeutically active. Furthermore, quercetin dose-dependently inhibited an inflammatory signal TNF-α-dependent NFκB activation.

Conclusions. Our data suggest that rutin acted as a quercetin deliverer to the large intestine and its anti-inflammatory action in TNBS-induced colitis rats may be through quercetin-mediated inhibition of TNF-α-induced NFκB activation.

KEY WORDS: inflammatory bowel disease; nuclear factor kappa B; quercetin; quercetin deliverer; rutin.

INTRODUCTION

A large number of flavonoids, mostly *O*-glycosides, are polyphenolic compounds of natural origin that are present in most fruits and vegetables. The average intake of the compounds by humans on a normal diet is more than 1 g per day. Although flavonoids are devoid of classical nutritional value, they are increasingly viewed as beneficial dietary components that possess antiallergic, anti-inflammatory, antiproliferative, and anticarcinogenic properties (1). It was reported that flavonoids act as potential protectors against human diseases such as coronary heart disease, cancers, and inflammatory bowel disease (IBD) (2,3).

Inflammatory bowel disease, ulcerative colitis, and Crohn's disease are chronic inflammatory conditions characterized by up-regulated pro-inflammatory mediators [including nuclear factor kappa B (NFκB] and pro-inflammatory cytokines (e.g., TNF-α, IL-1β) and dysregulated immune responses resulting in tissue damage (4). It is known that expression of TNF-α, which strongly activates NFκB, is itself up-regulated by NFκB. This provides a positive autoregulatory loop that amplifies the inflammatory response and perpetuates chronic intestinal inflammation (5). For this reason, therapeutic intervention against TNF-α or NFκB activation has been used for treatment of IBD (6,7). In fact, inhibition of NFκB activity has been suggested to be a major component of the anti-inflammatory activity of glucocorticoids and 5-aminosalicylic acid (5-ASA), both of which are frequently used for treatment of chronic intestinal inflammation (8,9).

Because sugar is a highly hydrophilic molecule that could limit gastrointestinal absorption of a drug coupled to it and certain glycosidic linkages are susceptible to the colonic microbial glycosidases but not to the host (human) ones (10), glycosides of glucocorticoids and 5-ASA have been developed as potential colon-specific prodrugs of the drugs for

[1] Laboratory of Biomedicinal Chemistry, College of Pharmacy, Pusan National University, Jangjeon-Dong, Keumjung-Gu, Pusan 609-735, Republic of Korea.
[2] Urologic Oncology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland 20892, USA.
[3] Laboratory of Medicinal Chemistry, College of Pharmacy, Pusan National University, Busan, Republic of Korea.
[4] Cell and Cancer Biology Branch, National Cancer Institute, Rockville, Maryland 20850, USA.
[5] To whom correspodence should be addressed. (e-mail: jungy@pusan.ac.kr)

ABBREVIATIONS: DMSO, dimethyl sulfoxide; HIF-1α, hypoxia-inducible factor-1 alpha; IBD, inflammatory bowel disease; NFDL, NFκB-dependent luciferase; NFκB, nuclear factor kappa B; TNBS, 2,4,6-trinitrobenzene sulfonic acid; 5-ASA, 5-aminosalicylic acid.

treatment of IBD. The data in the reports demonstrate that the prodrugs of glucocorticoids and 5-ASA successfully deliver the drugs to the large intestine and the drugs are liberated at the target site resulting in reducing the systemic side effects and enhancing the therapeutic effect (11).

It is thought that ingested flavonoid glycosides are not easily absorbed in the gastrointestine due to their high hydrophilicity; consequently, a large fraction of them reaches the large intestine where they could be metabolized by microbial glycosidases to liberate their aglycones and affect the (patho)-physiology of the large intestine. In this study, we investigated the metabolic properties of orally administered rutin, the most common flavonoid glycoside in nature, *in vitro* and *in vivo*, the therapeutic effect of rutin in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis rats, and a potential molecular mechanism of the therapeutic action of rutin. Our data demonstrate that rutin reached the large intestine without significant chemical and biochemical loss in the upper intestine and was rapidly deglycosylated to liberate quercetin in the large intestine. Orally administered rutin remarkably ameliorated TNBS-induced colitis rats and was as effective as sulfasalazine, a colon-specific prodrug of 5-ASA, which is most widely used for treatment of IBD (12). Furthermore, our results suggest that quercetin, the aglycone of rutin, was an active participant in rutin-mediated amelioration of TNBS-induced colitis rats and the therapeutic effect of quercetin was at least in part dependent on suppression of a major inflammatory signal, TNF-α-dependent NFκB activation.

MATERIALS AND METHODS

Rutin, quercetin, and 5-ASA were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Sulfasalazine and TNBS were purchased from Sigma (St. Louis, MO, USA). 3-Hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid were purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were reagent-grade commercially available products. Male Sprague–Dawley rats (240–260 g, 8 weeks old) were purchased from Daehan Biotec (Daegu, Korea) and housed in the animal care facility at Pusan National University, Busan, Korea.

High-Performance Liquid Chromatography Analysis

The concentration of rutin and quercetin was determined by reverse-phase high-performance liquid chromatography (HPLC, Gilson, Middleton, WI, USA) using a μBondapak C18 (3.9 × 300 mm, 10 μm; Waters, Milford, MA, USA) column with a guard column (3.9 × 20 mm, 5 μm). The mobile phase consisted of a 50/50 solution of methanol–0.067 M phosphate buffer (pH 4.5). Samples (20 μL) were injected and eluted with the mobile phase at a flow rate of 1.0 mL/min. The eluate was monitored at 257 nm at sensitivity of AUFS 0.001. The retention times of rutin and quercetin were 6.7 and 15.9 min, respectively.

Apparent Partition Coefficient

Apparent partition coefficients of rutin (10 μM) or quercetin (10 μM) were measured using a PBS/chloroform system (pH 6.8) as described previously (13).

Biochemical/Chemical Stability of Rutin and Quercetin

To determine chemical stability, either rutin (20 μM) or quercetin (10 μM) was incubated in pH 6.8 phosphate buffer and pH 1.2 hydrochloric acid buffer at 37°C for 24 h. At a predetermined time interval, rutin or quercetin in the buffers was analyzed by HPLC. To determine biochemical stability, a male Sprague–Dawley rat (240–260 g, 8 weeks old) was anesthetized by diethyl ether and a midline incision was made. Sections of the small intestine, cecum, and colon were collected separately and the contents from each section were diluted with pH 6.8 PBS. Rutin (200 μM) or quercetin (200 μM) was incubated in 1, 5, and 10% of the cecal contents at 37°C under nitrogen or 20% of the small intestinal contents at 37°C. An appropriate volume of the contents was taken at the predetermined time intervals, 20-fold diluted with methanol, and then centrifuged at 14,000 rpm. Rutin or quercetin in the supernatants was analyzed by HPLC.

Induction of Inflammation

Inflammation was induced by the method of Morris *et al.* (14) and Yano *et al.* (15). Briefly, before induction of colitis, rats were starved for 24 h but had free access to water. The rats were lightly anesthetized with ether. A rubber cannula (OD, 2 mm) was inserted rectally into the colon such that the tip was 8 cm proximal to the anus, approximately at the splenic flexture. TNBS dissolved in 50% (v/v) aqueous ethanol was instilled into the colon via the rubber cannula (15 mg/0.3 mL per rat).

Evaluation of TNBS-Induced Colitis

One day after induction of inflammation, drugs were administered orally or rectally once a day and the rats were sacrificed after the treatment for 6 days. A gross colonic damage score was calculated according to the criteria set forth previously (14,15). The modified scoring system is as follows: 0, normal appearance; 1, localized hyperemia but no ulcer; 2, linear ulcers without significant inflammation; 3, 2–4 cm site of inflammation and ulceration without scab; 4, serosal adhesion to other organs, 2–4 cm site of inflammation and ulceration with scab; 5, stricture, serosal adhesion involving several bowel loops, >4 cm site of inflammation and ulceration with scab. Four independent observers blinded to the treatment did the assessment of colonic damage score. Using the distal colon (4 cm), myeloperoxidase (MPO) activity was measured as described previously (16). One unit of MPO activity is defined as that degrading 1 μmol of peroxide per minute at 25°C.

Cells and Transient Transfection

Human colon epithelial cell lines HT-29, HCT116, and SW620 (American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (Biofluids, Rockville, MD, USA) supplemented with 10% fetal bovine serum (GIBCO, Rockville, MD, USA) and penicillin/streptomycin (Biofluids, Rockville, MD, USA). rh-TNF-α was purchased from R&D Systems (Minneapolis, MN, USA).

For transient transfection of NFκB-dependent luciferase (NFDL) plasmid, cells were plated in 12-well plates to be 50–60% confluent on the day of transfection with 0.5 μg NFDL plasmid (a gift from Dr. M. Birrer, National Cancer Institute, Bethesda, MD, USA) and 4 ng cytomegalovirus (CMV) *Renilla* luciferase plasmid (Promega, Madison, WI, USA). Fugene (Roche, South San Francisco, CA, USA) or Lipofectamine 2000 (GIBCO) was used as a transfection reagent. Sixteen hours posttransfection, cells were treated with TNF-α in the presence of each reagent at the indicated concentrations (see figure legends). Cells were lysed 6 h later and luciferase activities were measured and normalized to CMV *Renilla* luciferase activities using a Dual-Luciferase Assay Kit (Promega).

Western Blot

Cells were lysed and nuclear and cytosolic extracts were prepared as described (17). Cell lysates were electrophoretically separated using 4–20% gels (BioRad, Hercules, CA, USA). Proteins were transferred to nitrocellulose membranes (Protran, Schleicher & Schuell, Keene, NH, USA) and IκBα proteins were detected in cytosolic extracts using polyclonal anti-IκBα antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and p65 was detected in nuclear extracts using polyclonal anti-p65 antibody (Santa Cruz Biotechnology). Peroxidase-conjugated anti-rabbit secondary antibody (Amersham Life Science, Piscataway, NJ, USA) was used at a dilution of 1:1000. Signals were visualized using the SuperSignal chemiluminescence substrate (Pierce, Rockford, IL, USA).

DNA-Binding Assay and IL-8 ELISA

For DNA-binding assay, cell extracts were obtained by nuclear extraction as described above. Quercetin at various concentrations was incubated with the cell extracts for 30 min at 37°C. DNA-binding activity of p65 and hypoxia-inducible factor-1 alpha (HIF-1α) was measured with 5 μg of cell lysates using TransAM NFκB kit or TransAM HIF-1α kit (Active Motif, Carlsbad, CA, USA). For IL-8 ELISA, cells were treated with quercetin in the presence or absence of TNF-α for 8 h and immunoreactive IL-8 protein in cellular supernatants was measured using a Quantikine IL-8 ELISA kit (R&D Systems).

RESULTS

Rutin was Deglycosylated to Quercetin in the Cecal Contents but Stable in the Contents of the Upper Intestine

Due to the hydrophilicity of rutin, the quercetin glycoside, and susceptibility of the glycosidic linkage to microbial glycosidases in the colon (10), it was thought that oral administration of rutin, a glycoside of quercetin, could deliver its aglycone quercetin to the large intestine by its poor absorption in the upper intestine and its deglycosylation to quercetin in the large intestine. We investigated whether rutin had such properties. To examine whether the sugar residue in rutin increased hydrophilicity, apparent partition coefficients of rutin and its aglycone quercetin were compared using pH 6.8 PBS/chloroform system. Apparent partition coefficients of rutin and quercetin were 0.02 and 0.68, respectively, which suggests that the sugar residue rendered rutin more hydrophilic. In addition to greater hydrophilicity of rutin, rutin delivered to the large intestine should release quercetin from rutin at the site, whereas it was chemically and biochemically stable in the upper intestine. To test that, rutin was incubated in pH 1.2 and pH 6.8 buffer, the contents of the small intestine (20%), and the cecal contents (1, 5, and 10%) of rats. As shown in Fig. 1A, rutin was deglycosylated to liberate quercetin in the cecal contents, the rate of which is dependent on the concentration of the cecal contents, whereas it was chemically stable in the buffers up to at least 10 h (data not shown) and its glycosidic linkage was not cleaved in the contents of the small intestine. Interestingly, although the deglycosylation of rutin was apparently well correlated with the accumulation of quercetin in the 1% cecal contents, the level of quercetin increased and then began to decrease about 2 h after incubation of rutin in the 5% and about 1.5 h after that in the 10% cecal contents, resulting in the explicit two phases in the change of the quercetin level by deglycosylation of rutin. The result suggests that quercetin was also metabolized in the cecal contents; thus, quercetin alone was incubated in the cecal contents (1, 5, and 10%) and its concentration was measured at the predetermined time intervals. As shown in Fig. 1B, quercetin disappeared in the cecal contents, which was accelerated as concentration of the cecal contents increased. This indicates that the accumulation of quercetin liberated from rutin was shown by the net result of the liberation of quercetin from rutin and the disappearance of quercetin in the cecal contents.

Quercetin as Well as Rutin was Metabolized Enzymatically in the Cecal Contents

Because the metabolisms of foreign materials in the large intestine are usually carried out by microbial enzymes (18), we examined whether the disappearance of rutin and quercetin required an enzymatic action. Either rutin or querectin was incubated in pH 6.8 buffer, and the autoclaved and nonautoclaved 10% cecal contents and the rates of disappearance were compared. As shown in Fig. 2A and B, the disappearance of rutin or quercetin in the autoclaved cecal contents was markedly delayed compared with that in the nonautoclaved cecal contents. This suggests that both rutin and quercetin were metabolized by enzyme(s) in the cecal contents. Nonetheless, unlike rutin whose disappearance was mostly blocked by autoclaving the cecal contents, the disappearance of quercetin in the autoclaved cecal contents was delayed but still occurred faster than that in the buffer. Because it was reported that colonic inflammation changes the activities of microbial enzymes involved in the metabolisms of drugs in the large intestine (19), we examined whether induction of inflammation affected deglycosylation of rutin and subsequent accumulation of quercetin. Rutin was incubated in the cecal contents (1, 5, and 10%) obtained from TNBS-induced colitis rats. As shown in Fig. 2C, induction of inflammation gave no significant difference in the metabolisms of rutin and quercetin compared with those in the cecal content of the normal rats in Fig. 1A.

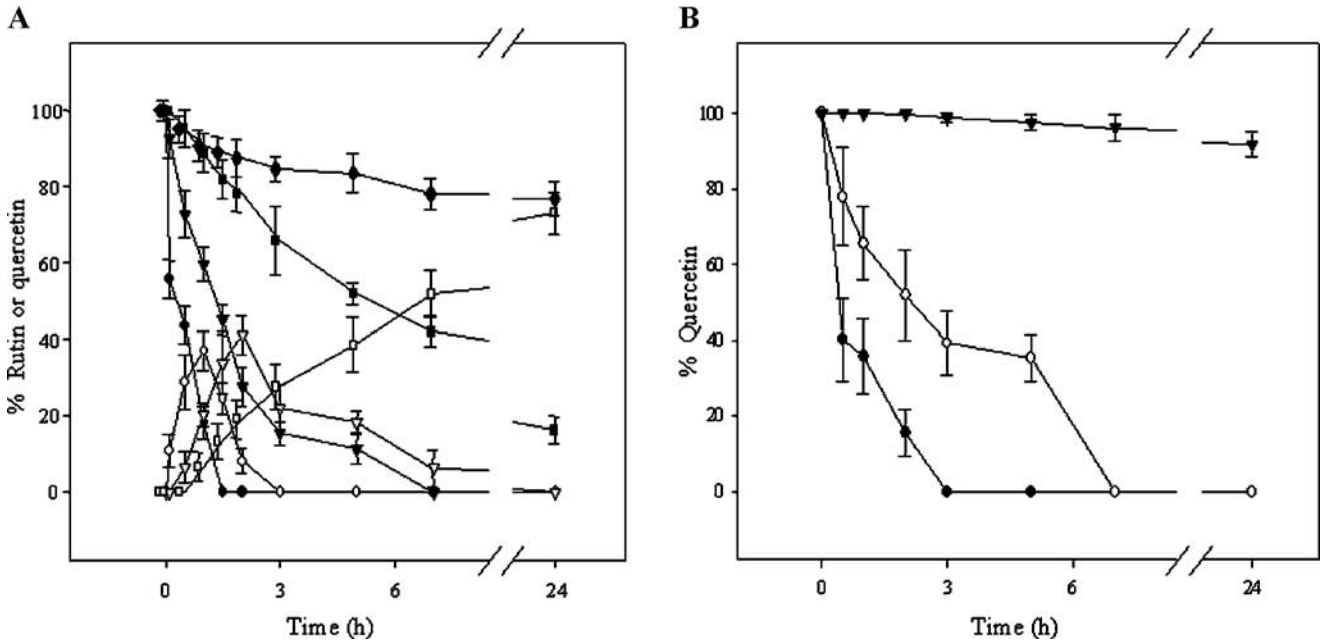

Fig. 1. Rutin was deglycosylated to quercetin in the cecal contents but stable in the contents of the upper intestine. (A) Rutin (200 μM) was incubated in the contents of the small intestine (20%) and the cecal contents (1, 5, and 10%) of rats. Appropriate volume of samples was taken at the time intervals indicated in the figure, 20-fold diluted with methanol, and centrifuged. Rutin and quercetin in the supernatants (20 μL) were analyzed by HPLC. ♦, Rutin in the 20% contents of the small intestine; ●, rutin in the 10% cecal contents; ○, quercetin liberated from rutin in the 10% cecal contents; ▼, rutin in the 5% cecal contents; ▽, quercetin liberated from rutin in the 5% cecal contents; ■, rutin in 1% the cecal contents; □, quercetin liberated from rutin in the cecal 1% contents. (B) Quercetin (200 μM) alone was incubated in the cecal contents (1, 5, and 10%). Appropriate volume of samples was taken at the time intervals indicated in the figure, 20-fold diluted with methanol, and centrifuged. Quercetin in the supernatants (20 μL) was analyzed by HPLC. ●, quercetin in the 10% cecal contents; ○, quercetin in the 5% cecal contents; ▼, quercetin in the 1% cecal contents. Data represent mean ± SD (n = 5).

Quercetin was Accumulated in the Inflamed Large Intestine After Oral Administration of Rutin

Although our *in vitro* data suggest that orally administered rutin would arrive and be deglycosylated to liberate quercetin in the large intestine, it still remained unclear whether, after oral administration, rutin delivered to the large intestine was enough to accumulate quercetin in the large intestine. To examine this, we administered rutin orally (10 mg/kg) to TNBS-induced colitis rats and concentrations of both rutin and quercetin were measured in the proximal small intestine, the distal small intestine, the cecum, and the colon 3, 5, and 7 h after oral administration. As shown in Fig. 3A and B, quercetin was not detected in the small intestine and rutin was observed in the large intestine only in the sample taken 5 h after oral administration, implying no deglycosylation of rutin in the upper intestine and rapid deglycosylation of rutin in the large intestine, which is in parallel with *in vitro* results. Moreover, most of rutin moved down through the small intestine to the large intestine until 7 h after oral administration and was deglycosylated to accumulate quercetin up to about 220 μM in the large intestine.

Oral Administration of Rutin is as Effective as that of Sulfasalazine in the Amelioration of Rat Colitis Induced by TNBS

Flavonoids including rutin are known to have a variety of biological activities including anti-inflammatory activity (20). We wished to confirm the anti-inflammatory effect of rutin and compare the effect of rutin with that of sulfasalazine, the most widely used therapeutics for IBD, using TNBS-induced colitis rats. Rutin (10 mg/kg) was administered orally to TNBS-induced colitis rats once a day starting from 1 day after induction of inflammation. Colonic damage score and MPO activity, an indicator of neutrophil infiltration, were determined after treatment with rutin for 6 days. The same experiment was done with sulfasalazine (30 mg/kg). As shown in Fig. 4A, in which colonic damage scores represent the extent of colonic injury by TNBS-induced inflammation, the normal colon showed no damage, but the control colon, the inflamed colon without treatment with rutin, was severely damaged, showing a scab formed by the hemorrhagic necrosis of the mucosa, stricture, and extensive serosal adhesion to other organs. Oral administration of rutin significantly healed the damaged colon and, furthermore, was as effective as sulfasalazine. Figure 4B shows that the level of MPO activity in the distal colon markedly increased by induction of inflammation and that treatment with rutin lowered the level of MPO activity to about 55% of the control. The effectiveness of rutin was similar to that of sulfasalazine, which is in accordance with the recovery of the colonic damage.

Quercetin was Therapeutically Active

Our *in vitro* results show that rutin delivered to the large intestine was rapidly deglycosylated to liberate quercetin; moreover, quercetin was extensively metabolized in the large

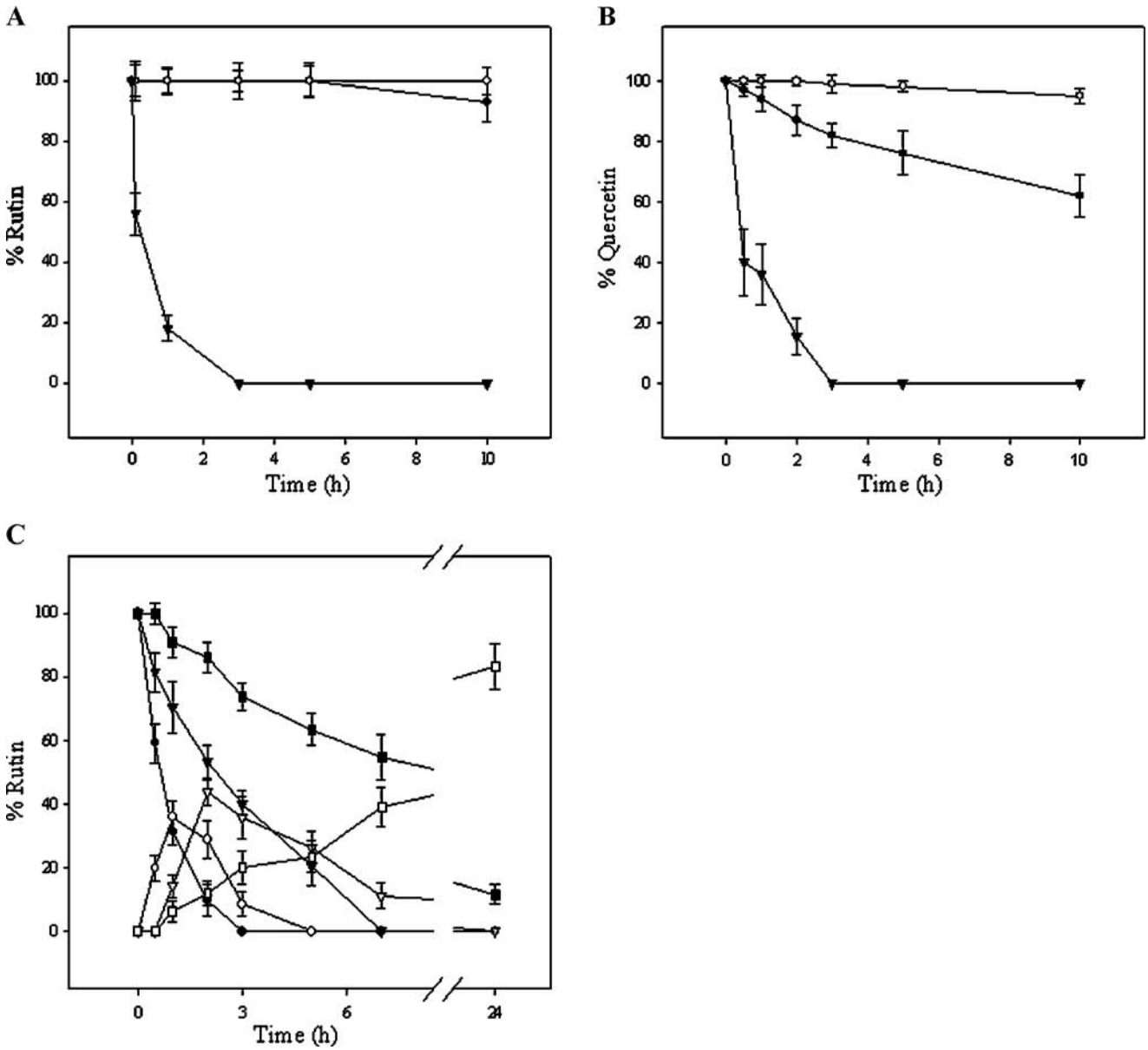

Fig. 2. Quercetin as well as rutin was metabolized enzymatically in the cecal contents. (A) Rutin was incubated in the autoclaved cecal contents (10%), nonautoclaved cecal contents (10%), and pH 6.8 buffer. Appropriate volume of samples was taken at the time intervals indicated in the figure, 20-fold diluted with methanol, and centrifuged. Rutin in the supernatants (20 μL) was analyzed by HPLC. ●, Rutin in the autoclaved cecal contents; ▼, rutin in the nonautoclaved cecal contents; ○, rutin in pH 6.8 buffer. (B) Quercetin was incubated in the autoclaved cecal contents (10%), nonautoclaved cecal contents (10%), and pH 6.8 buffer and analyzed as in A. ●, Quercetin in the autoclaved cecal contents; ▼, quercetin in the nonautoclaved cecal contents; ○, quercetin in pH 6.8 buffer. (C) Rutin was incubated with the cecal contents (1, 5, and 10%) of TNBS-induced colitis rats. Appropriate volume of samples was taken at the time intervals indicated in the figure, 20-fold diluted with methanol, and centrifuged. Rutin and quercetin in the supernatants (20 μL) were analyzed by HPLC. ●, Rutin in the cecal 10% contents; ▽, quercetin liberated from rutin in the 10% cecal contents; ▼, rutin in the 5% cecal contents; ▽, quercetin liberated from rutin in the 5% cecal contents; ■, rutin in the 1% cecal contents; □, quercetin liberated from rutin in the cecal 1% contents. Data represent mean ± SD (n = 5).

intestine, which is in agreement with previous reports (21,22). This suggests that quercetin liberated from rutin and/or its colonic metabolites may play a role in the amelioration of the colitis by orally administered rutin. To test this possibility, a 300-μL aliquot of quercetin (10, 25, 50, and 100 μM), 3-hydroxyphenylacetic acid (50 μM) or 3,4-dihydroxyphenylacetic acid (50 μM) in pH 6.8 PBS buffer was administered directly to the inflamed site through rectal route once a day starting from 1 day after induction of inflammation by TNBS. 3-Hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid are major colonic metabolites of quercetin (21). Colonic damage score and MPO activity were determined after intracolonic treatment with each drug for 6 days. The same experiment was done with a 300-μL aliquot of 20 mM sodium 5-aminosalicylate, the active ingredient of sulfasalazine, to compare the therapeutic activity with quercetin. The dose of

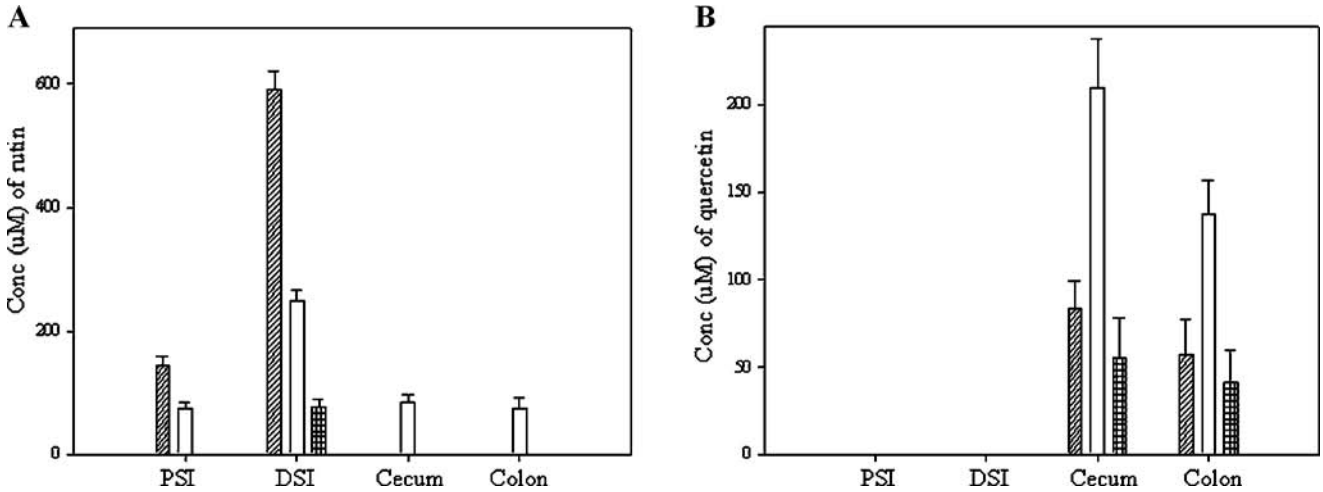

Fig. 3. Quercetin was accumulated in the inflamed large intestine after oral administration of rutin. (A). Rutin (10 mg/kg) was administered orally to the TNBS-induced colitis rats. The rats were sacrificed 3, 5, and 7 h after oral administration. The contents of the proximal small intestine (PSI), distal small intestine (DSI), cecum, and colon were collected separately, 20- to 40-fold diluted with methanol, and centrifuged. Rutin in the supernatants was analyzed by HPLC. ▨, rutin after 3 h; □, rutin after 5 h; ▦, rutin after 7 h. (B) The same experiment was done as in A. Quercetin in the supernatants was analyzed by HPLC. ▨, quercetin after 5 h; ▦, quercetin after 5 h; ▦, quercetin after 7 h. Data represent mean ± SD (n = 5).

5-ASA was decided based on the report demonstrating that 5-ASA (5-ASA + *N*-acetyl-5-ASA) concentration in the fecal water after oral administration of sulfasalazine in human is 5–30 mM (23). As shown in Fig. 5A, intracolonic treatment with quercetin healed the colonic damage by TNBS-induced inflammation in a dose-dependent manner and intracolonic treatment with 25 μM quercetin (300 μL) was as effective as that with 20 mM 5-ASA (300 μL). In contrast, no therapeutic effect was shown by intracolonic treatment with each metabolite. In accordance with these data, Fig. 5B shows that intracolonic treatment with quercetin or 5-ASA lowered the level of MPO activity in the distal colon and 25 μM quercetin (300 μL) and 20 mM 5-ASA (300 μL) were equally effective in lowering the level of MPO activity, whereas intracolonic treatment with each metabolite had no significant effect on the level of MPO activity.

Quercetin Inhibited TNF-α-Induced NFκB Activation in Human Colon Epithelial Cell Lines

As demonstrated, quercetin released from rutin showed the ability to alleviate the colonic inflammation. We speculated that a molecular mechanism underlies the quercetin-mediated therapeutic effects. The pro-inflammatory cytokine TNF-α is up-regulated in gut inflammation (24) and TNF-α-induced NFκB activity plays a central role in the production of pro-inflammatory mediators involved in progression of gut inflammation (5); thus we examined the effects of quercetin on NFκB activation by TNF-α. We transfected human colon epithelial cells HCT116, SW620, and HT-/.29 with an NFDL reporter gene in combination with an internal standard CMV *Renilla*Quercetin was accumulated in the inflamed luciferase plasmid. Cells were then treated with TNF-α in the presence

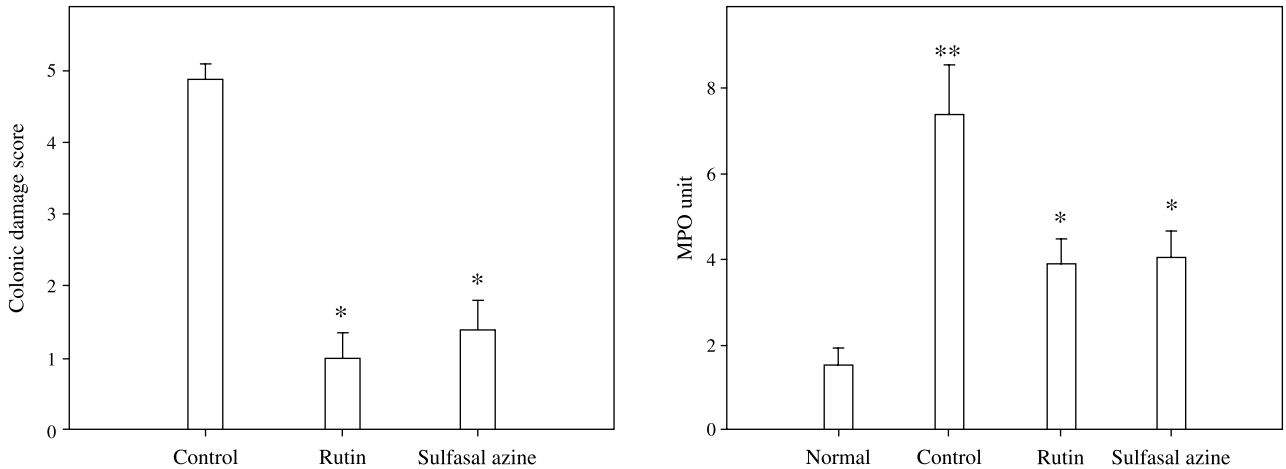

Fig. 4. Oral administration of rutin is as effective as that of sulfasalazine in the amelioration of rat colitis induced by TNBS. (A) Rutin (10 mg/kg) or sulfasalazine (30 mg/kg) was administered orally to the TNBS-induced colitis rats once a day starting from 1 day after induction of inflammation and the rats were sacrificed after treatment with the drugs for 6 days. The colonic damage scores were calculated according to the criteria described under Materials and Methods. (B) The same experiment was done as in A. Using the distal colon (4 cm), MPO activities were measured according to the criteria described under Materials and Methods. Data represent mean ± SD (n = 5).

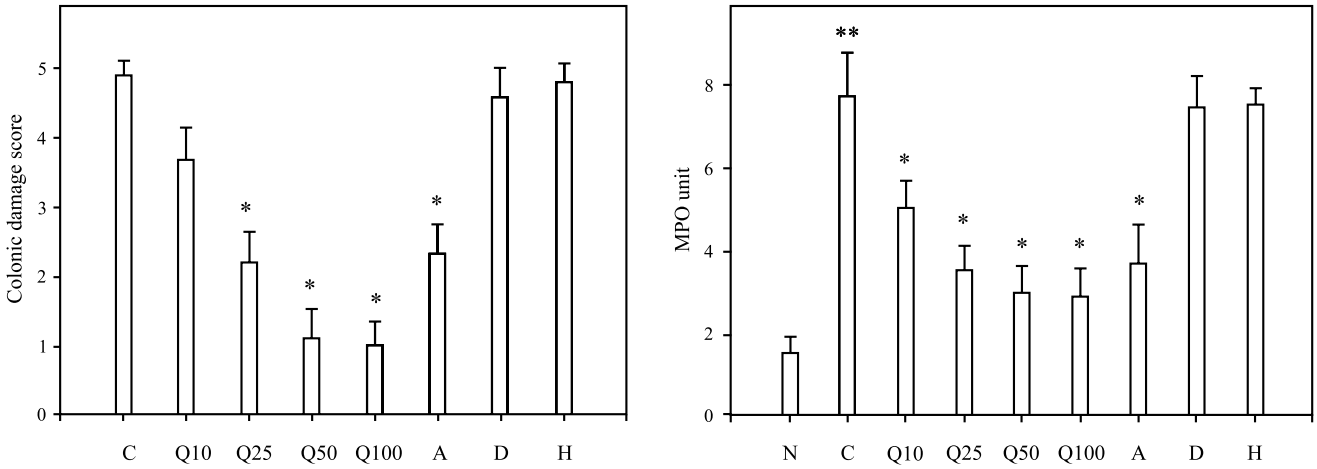

Fig. 5. Quercetin was therapeutically active. (A) A 300-μL aliquot of quercetin (10, 25, 50, and 100 μM), sodium 5-aminosalicylate (20 mM), 3,4-dihydroxyphenylacetic acid (50 μM), or 3-hydroxyphenylacetic acid (50 μM) in pH 6.8 PBS buffer was administered rectally to TNBS-induced colitis rats once a day starting from 1 day after induction of inflammation and the rats were sacrificed after treatment with each drug for 6 days. The colonic damage scores were calculated according to the criteria described under Materials and Methods. C, control; Q10, 10 μM quercetin, Q25, 25 μM quercetin; Q50, 50 μM quercetin; Q100, 100 μM quercetin; A, 20 mM sodium 5-aminosalicylate; D, 50 μM 3,4-dihydroxyphenylacetic acid; H, 50 μM 3-hydroxyphenylacetic acid. (B) The same experiment was done as in A. Using the distal colon (4 cm), MPO activities were measured according to the criteria described under Materials and Methods. N, normal; C, control; Q10, 10 μM quercetin, Q25, 25 μM quercetin; Q50, 50 μM quercetin; Q100, 100 μM quercetin; A, 20 mM sodium 5-aminosalicylate; D, 50 μM 3,4-dihydroxyphenylacetic acid; H, 50 μM 3-hydroxyphenylacetic acid. Data represent mean ± SD (n = 5).

or absence of quercetin (10–100 μM). As shown in Fig. 6A and B, although stimulation of cells with TNF-α markedly induced NFDL expression, quercetin inhibited NFDL expression by the cytokine in a dose-dependent manner. In contrast, neither rutin (10–100 μM) nor vehicle (dimethyl sulfoxide, DMSO) inhibited TNF-α-mediated NFDL expression (data not shown). To confirm that quercetin inhibited the NFκB pathway, we examined whether quercetin interfered with expression of IL-8, a neutrophil-attracting chemokine, in HT-29 cells. TNF-α up-regulates IL-8 through NFκB in these cells (25). We treated HT-29 cells with TNF-α in the presence or absence of quercetin at various concentrations. As shown in Fig. 6C, up-regulation of IL-8 by TNF-α was attenuated by quercetin in a dose-dependent manner.

Quercetin Inhibited TNF-α-Induced NFκB Activation by Interfering with DNA-Binding Activity of the NFκB p65 (RelA)

Because IκBα degradation in the cytosol and accumulation of p65 in the nucleus is critical for TNF-α-mediated NFκB activation (26), we investigated whether quercetin inhibited TNF-α-mediated NFκB activation by intervening in these processes. Human colon epithelial cells were incubated with TNF-α in the presence of varying concentrations of quercetin and lysed to obtain cytosolic and nuclear extracts. Western blotting was performed to analyze IκBα levels in the cytosol and p65 levels in the nucleus. As shown in Fig. 7A, stimulation of cells with the cytokine resulted in degradation of IκBα in the cytosol and accumulation of p65 in the nucleus. Pretreatment with quercetin did not affect the processes for NFκB activation by TNF-α. Because DNA-binding activity of transcription factors is important for their transcriptional activity (27), we hypothesized that quercetin might inhibit the DNA-binding activity of p65. To test this hypothesis, various concentrations of quercetin were added to nuclear extracts derived from TNF-α-treated HT-29 cells and the DNA-binding activity of p65 was measured using TransAM p65 Transcription Assay Kit. As shown in Fig. 7B, stimulation with TNF-α increased the DNA-binding activity of p65 up to 9-fold. Consistent with our hypothesis, quercetin interfered with the binding activity of p65 to the NFκB consensus DNA motif in a dose-dependent manner. Vehicle (DMSO) did not interfere with the DNA-binding activity of p65 (data not shown). Similar results were observed in HCT116 and SW620 cells (data not shown). To examine whether the effect of quercetin was specific for NFκB, quercetin (100 μM) was added to the nuclear extract from iron-chelator-treated HT-29 cells and the DNA-binding ability of HIF-1α in the nuclear extracts was measured using TransAM HIF-1α Transcription Assay Kit. As shown in Fig. 7C, in contrast to p65, the DNA binding of HIF-1α was not affected by quercetin.

DISCUSSION

In this study, we demonstrate that rutin acted as a quercetin deliverer to the large intestine and quercetin liberated from rutin ameliorated TNBS-induced colitis rats by at least partly inhibiting TNF-α-dependent NFκB activation.

Consistent with previous reports (10), our data demonstrate that rutin, a glycoside of quercetin, was deglycosylated to liberate its aglycone quercetin in the large intestine but not in the small intestine. In addition to the data, the results showing that rutin was chemically stable in pH 6.8 and pH 1.2 buffer and its hydrophilicity increased by the sugar residue imply that a large fraction of rutin administered orally would be delivered to and liberate quercetin in the large intestine due to poor systemic absorption and no significant chemical/biochemical loss in the upper intestine. These *in vitro* results

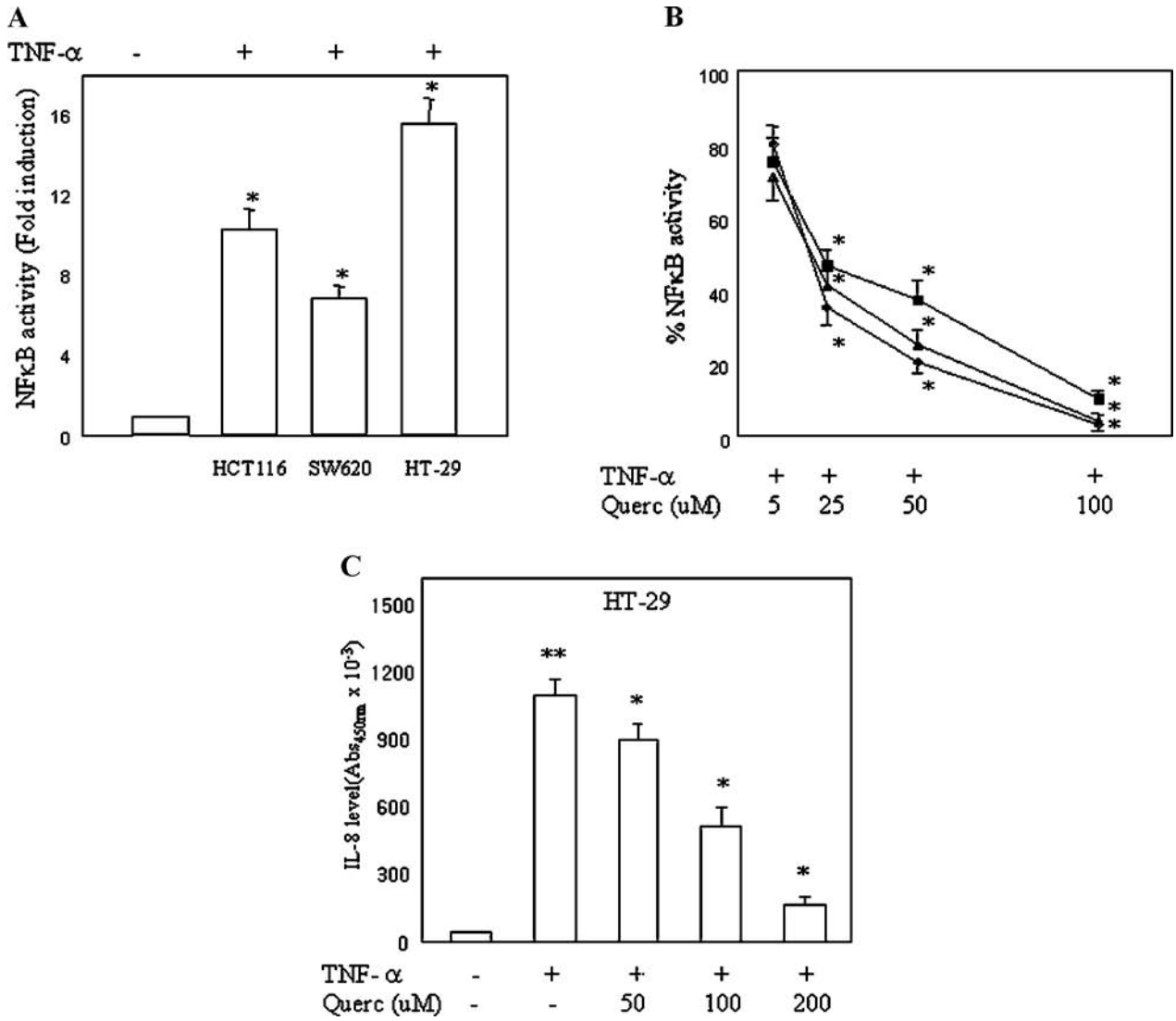

Fig. 6. Quercetin inhibited TNF-α-induced NFκB activation in human colon epithelial cell lines. (A) Cells were cotransfected with NFDL plasmid (0.4 μg) and CMV *Renilla* luciferase plasmid (4 ng) and subsequently treated with TNF-α (10 ng/mL) for 6 h. Reporter activities were measured and normalized to CMV *Renilla* luciferase activity. (B) The same experiment as in A was done in the presence or absence of various concentrations of quercetin (querc). ●, HCT116; ▲, SW620; ■, HT-29. (C) HT-29 cells were treated with TNF-α (10 ng/mL) in the presence or absence of various concentrations of quercetin (querc). Immunoreactive IL-8 was measured in the cellular supernatants using an IL-8 ELISA kit. The level of IL-8 in each cellular supernatant is represented by absorbance at 450 nm normalized to the total cellular protein. Data represent mean ± SD (n = 3).

are correlated with the *in vivo* data showing that a significant amount of quercetin was accumulated in the large intestine, whereas no quercetin was detected in the small intestine after oral administration of rutin. Because our data also showed that induction of inflammation, which reportedly affects the metabolism of drugs in the large intestine (19), made no big difference in deglycosylation of rutin and subsequent accumulation of quercetin, rutin is likely to act as a useful quercetin deliverer regardless of inflammation in the large intestine.

Liberation of quercetin from rutin seems to occur by microbial glycosidases in the large intestine. This is based on other reports (22,28) and our observation that the autoclaved cecal contents lost its ability to deglycosylate rutin to quercetin. Unlike rutin that is mostly metabolized by enzyme(s), quercetin is thought to be metabolized not only enzymatically but also nonenzymatically in the cecal contents, as the disappearance of quercetin in the autoclaved cecal contents was markedly delayed but still occurred faster than that in pH 6.8 buffer. The enzymatic metabolism of quercetin in the cecal contents is in agreement with previous reports (29,21). Although we do not rule out the existence of a heat-resistant enzyme in the autoclaved cecal contents that is still able to metabolize quercetin, the nonenzymatic metabolism of quercetin may be due to conversion of quercetin to reactive electrophilic *o*-quinones, which could form adducts with protein, DNA, and RNA (30).

As reported by Cruz *et al.* (31), our data demonstrate that oral administration of rutin ameliorated TNBS-induced colitis rats as evaluated by measurement of MPO activity and colonic damage score. We suggest that the therapeutic effect of rutin on the rat colitis was exerted by quercetin liberated from rutin in the large intestine. This is supported by the

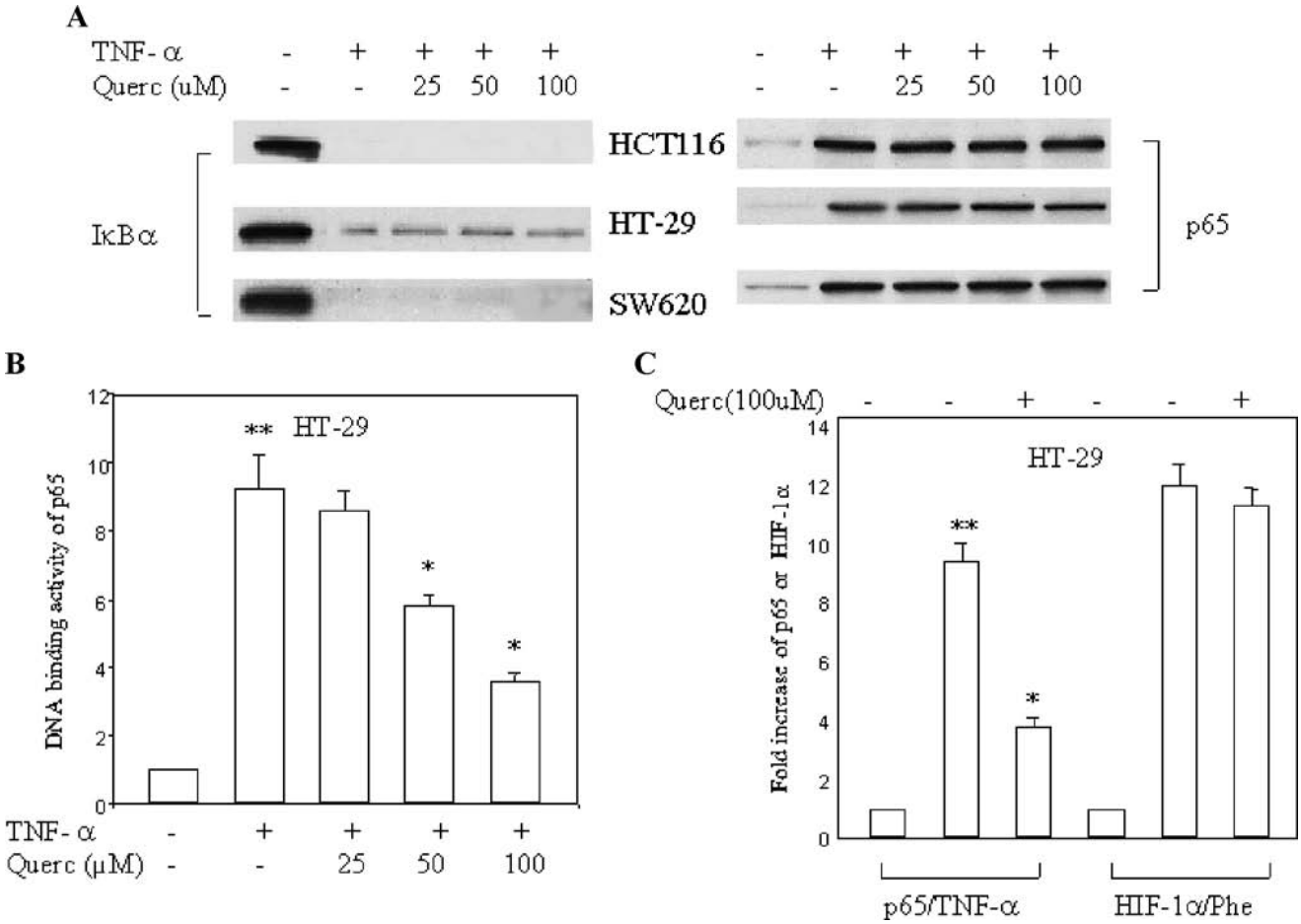

Fig. 7. Quercetin inhibited TNF-α-induced NFκB activation by interfering with DNA-binding activity of the NFκB p65 (RelA). (A) Cells were treated with TNF-α (10 ng/mL) in the presence or absence of various concentrations of quercetin (querc) and IκBα or p65 protein levels were monitored in cytosolic or nuclear extracts, respectively. (B) HT29 cells were left untreated or treated with TNF- for 20 min and lysed to obtain nuclear extracts. Various concentrations of quercetin were added to the nuclear extracts from TNF-α-treated cells and incubated for 30 min at room temperature. DNA-binding activity of p65 was measured using 5 μg of the nuclear extracts. (C) HT-29 cells were treated with either TNF-α for 20 min or an iron chelator, phenanthroline (Phe, 100 μM), for 4 h and lysed to obtain the nuclear extracts. Quercetin (querc) was added to the nuclear extracts to afford 100 μM quercetin and incubated for 30 min. DNA-binding activity of p65 and HIF-1α was measured using 5 μg of each nuclear extract. Data represent mean ± SD (n = 3).

following data: 1) orally administered rutin accumulated a significant amount of quercetin in the large intestine, which was seen for at least 4 h; 2) intracolonic treatment with quercetin healed the colonic damage and lowered MPO activity in the inflamed colon in a dose-dependent manner; and 3) no therapeutic effect was shown by intracolonic treatment with two major metabolites that may be produced by the metabolism of quercetin in the large intestine. However, we do not absolutely rule out an involvement of other minor metabolites of quercetin and rutin in the therapeutic effect by oral administration of rutin.

In our experimental condition, oral administration of rutin (10 mg/kg) was as effective in amelioration of TNBS-induced colitis rats as that of sulfasalazine (30 mg/kg), which is the most widely used prodrug of 5-ASA for treatment of IBD. Although we did not determine the concentration of 5-ASA in the large intestine after oral administration of sul fasalazine, it is thought that the concentration of 5-ASA provided from sulfasalazine administered orally was much greater than that of quercetin provided from rutin administered orally, based on the finding of no metabolism of 5-ASA in the colonic contents along with administration of sulfasalazine at threefold higher dose (32). This suggests that quercetin may be more potent than sulfasalazine in treatment of IBD. This argument is supported by the data showing that intracolonic treatment with 25 μM quercetin (300 μL) was as effective as that with 20 mM 5-amnosalicylic acid (300 μL), the active ingredient of sulfasalazine. However, a more elaborate experiment with a range of 5-ASA doses is required for a better comparison of the potency. Considering that quercetin is a relatively safe natural product (as in the term "a dietary flavonoid"), whereas long-term treatment with sulfasalazine causes severe to mild adverse effects in a large percentage of IBD patients (12), it is very likely that quercetin is a lead compound for development of a new pharmacological agent for treatment of IBD.

Consistent with other reports demonstrating that quercetin inhibits NFκB activation and induction of its target genes in many cell lines including lung and endothelial cells (33), our data demonstrate that quercetin dose-dependently

inhibited TNF-α-induced NFκB activation in the human colon cells as illustrated by the data showing that quercetin inhibited TNF-α-mediated expression of NFDL reporter gene and induction of IL-8, a target gene of NFκB, in a dose-dependent manner. Because TNF-α-induced NFκB activation is a major inflammatory pathway playing a critical role in the development and maintenance of IBD (5), this result at a molecular level suggests that the clinically beneficial effect of quercetin on TNBS-induced colitis rats was through quercetin-mediated inhibition of TNF-α-induced NFκB activation. The connection between the clinical effect and the molecular mechanism is further supported by demonstrating that quercetin-mediated attenuation of IL-8 (a potent neutrophil-attracting chemokine) induction was well correlated with reduction of MPO activity (an indicator for recruitment of neutrophil) in the distal colon.

Contrary to previous papers (34,35), treatment with quercetin did not prevent the processes of TNF-α-induced NFκB activation, cytosolic IκBα degradation, and subsequent nuclear p65 accumulation in our experiments. The different responses we observed in the three human colonic cells used may be cell-type dependent. We suggest that quercetin suppresses TNF-α-induced NFκB activation by at least partly interfering with the binding of p65 to DNA, resulting in inhibition of the transcriptional activity of NFκB. This hypothesis is based on our finding that quercetin attenuated the DNA binding of p65 obtained from the nuclear extract of TNF-α-treated cells in a dose-dependent manner, although the dose response did not completely mirror that of quercetin-mediated inhibition of NFκB transcriptional activity. As discussed above, quercetin can be converted to the reactive electrophilic *o*-quinones, which could alkylate proteins. It should be noted that parthenolide, a sesquiterpene lactone, inhibits NFκB activation by preventing the DNA binding of p65, which occurs by parthenolide alkylating the thiol in p65 (36).

Our data in this report confirmed the previous reports (31,37) on the effectiveness of quercetin glycosides in experimental colitis and further provided the metabolic and pharmacological information underlying the clinical effect of rutin, which may be important for the development of a new therapeutic agent for the treatment of IBD. In our laboratory, we are currently investigating how to develop derivatives of quercetin that not only are resistant to the colonic metabolism but also effectively inhibit NFκB activation in human colon epithelial cells.

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REFERENCES

1. R. J. Nijveldt, E. van Nood, D. E. van Hoorn, P. G. Boelens, K. van Norren, and P. A. van Leeuwen. Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **74**(4):418–425 (2001).
2. P. C. Hollman and M. B. Katan. Absorption, metabolism and health effects of dietary flavonoids in man. *Biomed. Pharmacother.* **51**(8):305–310 (1997).
3. J. M. Gee and I. T. Johnson. Polyphenolic compounds: interactions with the gut and implications for human health. *Curr. Med. Chem.* **8**(11):1245–1255 (2001).
4. C. Fiocchi. Intestinal inflammation: a complex interplay of immune and nonimmune cell interactions. *Am. J. Physiol.* **273**(4 Pt 1):G769–G775 (1997).
5. M. F. Neurath, C. Becker, and K. Barbulescu. Role of NF-kappaB in immune and inflammatory responses in the gut. *Gut* **43**(6):856–860 (1998).
6. H. Ogata and T. Hibi. Cytokine and anti-cytokine therapies for inflammatory bowel disease. *Curr. Pharm. Des.* **9**(14):1107–1113 (2003).
7. C. K. Weber, S. Liptay, T. Wirth, G. Adler, and R. M. Schmid. Suppression of NF-kappaB activity by sulfasalazine is mediated by direct inhibition of IkappaB kinases alpha and beta. *Gastroenterology* **119**(5):1209–1218 (2000).
8. F. Yan and D. B. Polk. Aminosalicylic acid inhibits IkappaB kinase alpha phosphorylation of IkappaBalpha in mouse intestinal epithelial cells. *J. Biol. Chem.* **274**(51):36631–36636 (1999).
9. N. Auphan, J. A. DiDonato, C. Rosette, A. Helmberg, and M. Karin. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science* **270**(5234):286–290 (1995).
10. P. C. Hollman, M. N. Bijsman, Y. van Gameren, E. P. Cnossen, J. H. de Vries, and M. B. Katan. The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Radic. Res.* **31**(6):569–573 (1999).
11. V. R. Sinha and R. Kumria. Colonic drug delivery: prodrug approach. *Pharm. Res.* **18**(5):557–564 (2001).
12. B. Crotty and D. P. Jewell. Drug therapy of ulcerative colitis. *Br. J. Clin. Pharmacol.* **34**(3):189–198 (1992).
13. Y. J. Jung, J. S. Lee, H. H. Kim, Y. M. Kim, and S. K. Han. Synthesis and evaluation of 5-aminosalicyl-glycine as a potential colon-specific prodrug of 5-aminosalicylic acid. *Arch. Pharm. Res.* **21**(2):174–178 (1998).
14. G. P. Morris, P. L. Beck, M. S. Herridge, W. T. Depew, M. R. Szewczuk, and J. L. Wallace. Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology* **96**(3):795–803 (1989).
15. H. Yano, F. Hirayama, M. Kamada, H. Arima, and K. Uekama. Colon-specific delivery of prednisolone-appended alpha-cyclodextrin conjugate: alleviation of systemic side effect after oral administration. *J. Control. Release* **79**(1–3):103–112 (2002).
16. M. W. Son, J. I. Ko, H. M. Doh, W. B. Kim, T. S. Park, M. J. Shim, and B. K. Kim. Protective effect of taurine on TNBS-induced inflammatory bowel disease in rats. *Arch. Pharm. Res.* **21**(5):531–536 (1998).
17. N. C. Andrews and D. V. Faller. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.* **19**(9):2499 (1991).
18. R. R. Scheline. Metabolism of foreign compounds by gastrointestinal microorganisms. *Pharmacol. Rev.* **25**(4):451–523 (1973).
19. P. Marteau, P. Seksik, and F. Shanahan. Manipulation of the bacterial flora in inflammatory bowel disease. *Best Pract. Res., Clin. Gastroenterol.* **17**(1):47–61 (2003).
20. J. V. Formica and W. Regelson. Review of the biology of quercetin and related bioflavonoids. *Food Chem. Toxicol.* **33**(12):1061–1080 (1995).
21. A. M. Aura, K. A. O'Leary, G. Williamson, M. Ojala, M. Bailey, R. Puupponen-Pimia, A. M. Nuutila, K. M. Oksman-Caldentey, and K. Poutanen. Quercetin derivatives are deconjugated and converted to hydroxyphenylacetic acids but not methylated by human fecal flora *in vitro*. *J. Agric. Food Chem.* **50**(6):1725–1730 (2002).
22. A. R. Rechner, M. A. Smith, G. Kuhnle, G. R. Gibson, E. S. Debnam, S. K. Srai, K. P. Moore, and C. A. Rice-Evans. Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. *Free Radic. Biol. Med.* **36**(2):212–225 (2004).
23. L. A. Christensen, J. Fallingborg, B. A. Jacobsen, K. Abildgaard, H. H. Rasmussen, S. N. Rasmussen, and S. H. Hansen. Bioavailability of 5-aminosalicyclic acid from slow release 5-aminosalicyclic acid drug and sulfasalazine in normal children. *Dig. Dis. Sci.* **38**(10):1831–1836 (1993).
24. T. Hibi, N. Inoue, H. Ogata, and M. Naganuma. Introduction

and overview: recent advances in the immunotherapy of inflammatory bowel disease. *J. Gastroenterol.* **38**(Suppl. 15): 36–42 (2003).
25. S. W. Kim, S. C. Choi, E. Y. Choi, K. S. Kim, J. M. Oh, H. J. Lee, H. M. Oh, S. Kim, B. S. Oh, K. C. Kimm, M. H. Lee, G. S. Seo, T. H. Kim, H. C. Oh, W. H. Woo, Y. S. Kim, H. O. Pae, D. S. Park, H. T. Chung, and C. D. Jun. Catalposide, a compound isolated from *Catalpa ovata*, attenuates induction of intestinal epithelial proinflammatory gene expression and reduces the severity of trinitrobenzene sulfonic acid-induced colitis in mice. *Inflamm. Bowel Dis.* **10**(5):564–572 (2004).
26. P. A. Baeuerle and D. Baltimore. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science* **242**(4878):540–546 (1988).
27. C. Kunsch, S. M. Ruben, and C. A. Rosen. Selection of optimal kappa B/Rel DNA-binding motifs: interaction of both subunits of NF-kappa B with DNA is required for transcriptional activation. *Mol. Cell. Biol.* **12**(10):4412–4421 (1992).
28. S. Baba, T. Furuta, M. Fujioka, and T. Goromaru. Studies on drug metabolism by use of isotopes: XXVII. Urinary metabolites of rutin in rats and the role of intestinal microflora in the metabolism of rutin. *J. Pharm. Sci.* **72**(10):1155–1158 (1983).
29. P. C. Hollman, M. vd Gaag, M. J. Mengelers, J. M. van Trijp, J. H. de Vries, and M. B. Katan. Absorption and disposition kinetics of the dietary antioxidant quercetin in man. *Free Radic. Biol. Med.* **21**(5):703–707 (1996).
30. T. Walle, T. S. Vincent, and U. K. Walle. Evidence of covalent binding of the dietary flavonoid quercetin to DNA and protein in human intestinal and hepatic cells. *Biochem. Pharmacol.* **65**(10):1603–1610 (2003).
31. T. Cruz, J. Galvez, M. A. Ocete, M. E. Crespo, L. H. F. Sanchez de Medina, and A. Zarzuelo. Oral administration of rutoside can ameliorate inflammatory bowel disease in rats. *Life Sci.* **62**(7):687–695 (1998).
32. S. Y. Zhou, D. Fleisher, L. H. Pao, C. Li, B. Winward, and E. M. Zimmermann. Intestinal metabolism and transport of 5-amino-salicylate. *Drug Metab. Dispos.* **27**(4):479–485 (1999).
33. K. Muraoka, K. Shimizu, X. Sun, T. Tani, R. Izumi, K. Miwa, and K. Yamamoto. Flavonoids exert diverse inhibitory effects on the activation of NF-kappaB. *Transplant. Proc.* **34**(4):1335–1340 (2002).
34. S. Y. Cho, S. J. Park, M. J. Kwon, T. S. Jeong, S. H. Bok, W. Y. Choi, W. I. Jeong, S. Y. Ryu, S. H. Do, C. S. Lee, J. C. Song, and K. S. Jeong. Quercetin suppresses proinflammatory cytokines production through MAP kinases and NF-kappaB pathway in lipopolysaccharide-stimulated macrophage. *Mol. Cell. Biochem.* **243**(1–2):153–160 (2003).
35. M. M. Mu, D. Chakravortty, T. Sugiyama, N. Koide, K. Takahashi, I. Mori, T. Yoshida, and T. Yokochi. The inhibitory action of quercetin on lipopolysaccharide-induced nitric oxide production in RAW 264.7 macrophage cells. *J. Endotoxin Res.* **7**(6):431–438 (2001).
36. A. J. Garcia-Pineres, V. Castro, G. Mora, T. J. Schmidt, E. Strunck, H. L. Pahl, and I. Merfort. Cysteine 38 in p65/NF-kappaB plays a crucial role in DNA binding inhibition by sesquiterpene lactones. *J. Biol. Chem.* **276**(43):39713–39720 (2001).
37. F. Sanchez de Medina, J. Galvez, J. A. Romero, and A. Zarzuelo. Effect of quercetin on acute and chronic experimental colitis in the rat. *J. Pharmacol. Exp. Ther.* **278**(2):771–779 (1996).