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Quercetin Inhibits TNF-Induced NF-κB Transcription Factor Recruitment to Proinflammatory Gene Promoters in Murine Intestinal Epithelial Cells[1,2]

Pedro A. Ruiz,[3] Annett Braune,[4] Gabriele Hölzlwimmer,[5] Leticia Quintanilla-Fend,[5] and Dirk Haller[3]*

[3]Else-Kroener-Fresenius-Center for Experimental Nutritional Medicine, Technical University of Munich, 85350 Freising-Weihenstephan, Germany; [4]Institute of Pathology, GSF Research Center, Munich-Neuherberg, Germany; and [5]German Institute of Human Nutrition, Intestinal Microbiology, 14558 Nuthetal, Germany

Abstract

Flavonoids may play an important role for adjunct nutritional therapy of chronic intestinal inflammation. In this study, we characterized the molecular mechanisms by which quercetin and its enteric bacterial metabolites, taxifolin, alphitonin, and 3, 4-dihydroxy-phenylacetic acid, inhibit tumor necrosis factor α (TNF)-induced proinflammatory gene expression in the murine small intestinal epithelial cell (IEC) line Mode-K as well as in heterozygous TNFΔARE/WT mice, a murine model of experimental ileitis. Quercetin inhibited TNF-induced interferon-γ-inducible protein 10 (IP-10) and macrophage inflammatory protein 2 (MIP-2) gene expression in Mode-K cells with effective inhibitory concentration of 40 and 44 μmol/L, respectively. Interestingly, taxifolin, alphitonin, and 3,4-dihydroxy-phenylacetic acid did not inhibit TNF responses in IEC, suggesting that microbial transformation of quercetin completely abolished its anti-inflammatory effect. At the molecular level, quercetin inhibited Akt phosphorylation but did not inhibit TNF-induced RelA/I-κB phosphorylation and IκB degradation or TNF-α-induced nuclear factor-κB transcriptional activity. Most important for understanding the mechanism involved, chromatin immunoprecipitation analysis revealed inhibitory effects of quercetin on phospho-RelA recruitment to the IP-10 and MIP-2 gene promoters. In addition, and consistent with the lack of cAMP response element binding protein (CBP)/p300 recruitment and phosphorylation/acetylation of histone 3 at the promoter binding site, quercetin inhibited histone acetyl transferase activity. The oral application of quercetin to heterozygous TNFΔARE/WT mice [10 mg/(d x kg body wt)] significantly inhibited IP-10 and MIP-2 gene expression in primary ileal epithelial cells but did not affect tissue pathology. These studies support an anti-inflammatory effect of quercetin in epithelial cells through mechanisms that inhibit cofactor recruitment at the chromatin of proinflammatory genes. J. Nutr. 137: 1208–1215, 2007.

Introduction

The flavonol quercetin is the most common flavonoid in nature and is often linked to sugars such as rutin (quercetin-3-rutinoside) and quercitrin (quercetin-3-rhamnoside) (1,2). Quercetin is reported to have antioxidant properties (3,4) associated with antithrombic, antihypertensive, anticarcinogenic and anti-inflammatory effects (5–7). In addition, quercetin inhibits a broad spectrum of protein kinases by its capability to compete with the binding of ATP at the nucleotide binding site (8). Interestingly, quercetin was the lead compound used to develop LY294002 and other inhibitors of phosphoinositide 3 (PI3)[6] kinase (9), which is involved in the activation of a variety of downstream kinases including the protein kinase B/Akt (10). Rutin and quercitrin exert intestinal anti-inflammatory effects in experimental models of rat colitis, the last being associated with loss of inducible nitric oxide synthase (iNOS) expression in dextran sodium sulfate-induced colitis (11,12). Quercetin itself mediated anti-inflammatory effects in paw edema induced by carrageenan in rats (13). It has been suggested that the protective effects of quercetin may be attributed to its antioxidant and free-radical scavenging properties, thereby improving the colonic oxidative stress mechanisms under the inflammatory status (14). In addition, quercetin has been shown

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* To whom correspondence should be addressed. E-mail: haller@wzw.tum.de.

[6] Abbreviations used: ChIP, chromatin immunoprecipitation; HAT, histone acetyl transferase; IBD, inflammatory bowel diseases; IEC, intestinal epithelial cell; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; IP3, phosphoinositide 3; IP-10, interferon-γ-inducible protein 10; MIP-2, macrophage inflammatory protein 2; CBP, cAMP response element binding protein; NF-κB, nuclear factor κB; SEAP, secreted alkaline phosphatase; TNF, tumor necrosis factor α.

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to inhibit both macrophage proliferation and activation in vitro by blocking the activation of lipopolysaccharide-induced nuclear factor κB (NF-κB) signaling (15). Although quercetin affects stress/cytokine-induced NF-κB and Akt signal transduction (16–18) and to some extent inhibits experimental colitis (15,19,20), the molecular mechanisms of this polyphenolic compound to inhibit epithelial cell activation under conditions of chronic intestinal inflammation is not yet well defined.

The presence of gastrointestinal infections, the genetic predisposition to dysregulated mucosal immune responses, and environmental triggers in developed countries represent etiologic factors for the development of ulcerative colitis and Crohn's disease, the 2 distinct pathologies of inflammatory bowel disease (IBD) (21–23). Despite the clinic development of specific biologic therapies in the last years (24), little is known about the anti-inflammatory effects of dietary components. Increased activity of the NF-κB transcription factor system has been documented in the intestinal epithelium of animal models for experimental colitis (25) and IBD patients (26–28); therefore, pharmacological blockade of the NF-κB signaling pathway may become particularly important in the treatment of chronic intestinal inflammation (29). Intestinal epithelial cells (IEC) adapt to a constant changing environment by processing the combined biological information of luminal enteric bacteria/nutritional factors (30) as well as host-derived immune signals (31–33); therefore, IEC become an excellent target cell type to assess the anti-inflammatory effects of dietary components on the host (34).

The treatment of a subset of IBD patients with monoclonal antibodies to tumor necrosis factor α (TNF) (infliximab) induced clinical remission of the inflammatory disease status (35), supporting the concept that TNF plays an important role in initiating and perpetuating NF-κB signaling and chronic intestinal inflammation (36,37). At the cellular level, TNF targets the TNF receptor 1 (TNFR1) to induce NF-κB RelA (Ser536) and IκBα (Ser32/34) phosphorylation, as well as IκBα ubiquitination and proteasomal degradation. Transcriptionally active NF-κB subunits such as RelA translocate to the nucleus to induce κB-dependent gene expression (38,39). Interestingly, the full activation of TNF-induced NF-κB activity and proinflammatory gene expression also requires additional mechanisms, including Akt serine-threonine kinase activation (40).

We have shown previously that NF-κB signal transduction and gene expression of the IFN-γ-inducible protein 10 (IP-10) were persistently active in primary IEC under conditions of experimental colitis (25). Although flavonoids targeted the NF-κB and Akt signaling pathways to inhibit TNF-induced IP-10 gene expression in IEC (41), the molecular mechanisms for the inhibitory effects of quercetin remained unclear. In this study, we demonstrated that quercetin inhibits NF-κB binding to the proinflammatory IP-10 and MIP-2 gene promoters, therefore further blocking cofactor recruitment and histone acetyl transferase (HAT) activity at the chromatin of these promoters. Interestingly, quercetin metabolites accruing from bacteria-mediated degradation in the intestinal tract (42,43) did not inhibit TNF-induced gene expression, suggesting limited biological function along the intestinal tract. To further validate the physiological relevance of the inhibitory effects of quercetin with respect to the TNF-induced IP-10 and MIP-2 gene expression, we orally administered quercetin [10 mg/(d × kg body wt)] to heterozygous TNFΔARE/WT mice. These mice lack the translational repression of TNF due to the absence of TNF adenosine- and uracil-rich elements in 3′-untranslated region of the TNF mRNA transcripts and, as a consequence, develop experimental ileitis (44,45).

Materials and Methods

Cell culture and treatments. The mouse IEC line Mode-K (passage 10–30) was grown to confluency in 6-well tissue culture plates (Cell Star, Greiner Bio-One) as previously described (30). Mode-K cells were stimulated with TNF (5 μg/L; R&D Systems) in the absence or presence of quercetin, taxifolin (both from Roth), alphitonin (kindly provided by Dr. A. Braune, German Institute of Human Nutrition, Intestinal Microbiology), and 3,4-dihydroxy-phenylacetic acid (Sigma-Aldrich) at a final concentration of 100 μmol/L. The chemical structures of quercetin and its metabolite products are shown in **Figure 1**. Dose-response experiments were performed with quercetin in a concentration range of 1–200 μmol/L incubating with TNF for 24 h. The effective inhibitory concentration of this compound was determined by calculating the inflection point of the inhibition curve. TNF-induced IP-10 and MIP-2 protein concentrations were blotted against the flavonoid concentration. Where indicated, we used pharmacological inhibitors including the NF-κB inhibitor PDTC, the PI3K/Akt inhibitor LY291002 (both from Sigma-Aldrich), and the p38 MAPK inhibitor SB203580 (20 μmol/L; Calbiochem, Merck Biosciences).

Oral application of quercetin to heterozygous TNFΔARE mice. Heterozygous TNFΔARE/WT and wild type (WT) mice at the age of 8 wk were orally fed with quercetin [10 mg/(d × kg body wt)] to the treatment group (Q, $n = 11$) and its dissolvent, propylene glycol, was fed alone via crop gavage to the control group (C, $n = 10$). All mice received a standard diet with low nitrosamine contents, composed of fatty acids, minerals, amino acids, vitamins, and crude nutrients such as starch and crude protein (R/M-H, ssniff). The mice were a generous gift from Dr. G. Kollias (Laboratory of Molecular Genetics, Hellenic Pasteur Institute, Athens, Greece). The animal-use protocols were approved by the Bavarian Animal Care and Use Committee (AZ 55.2-1-54-2531-74-06). Mice were killed after 10 wk of treatment at the age of 18 wk by cervical dislocation, and primary IEC were isolated from the ileum. Sections of the distal ileum were fixed in 10% neutral buffered formalin. The fixed tissue was embedded in paraffin and histopathological analysis was performed as previously described, at the Institute of Pathology (GSF Research Center) (46). In addition, we used the paraffin-embedded ileal tissue section to perform immunohistochemistry, using anti-RelA antibodies (Santa Cruz), according to the protocol of the manufacturer.

Isolation of primary ileal epithelial cells. Primary IEC from the ileal epithelium of quercetin and propylene glycol fed WT and TNFΔARE/WT were purified as previously described (25). Cell purity was assessed by determining the absence of $CD3^+$ T-cell contamination. Trypan blue exclusion confirmed the presence of at least 80% viable cells after the 2-h isolation procedure. Primary IEC from the ileum were collected in sample buffer for subsequent RNA isolation.

RNA isolation and real-time RT-PCR. RNA from purified native IEC was extracted using Trizol Reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Extracted RNA was dissolved

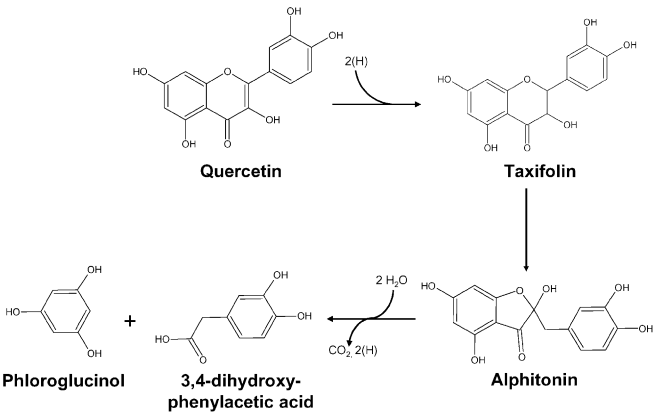

FIGURE 1 Chemical structures of quercetin and its bacterial metabolites.

in 20 μL water containing 0.1% diethyl-pyrocarbonate. Reverse transcription was performed from 1 μg total RNA. Real-time PCR was performed from 1 μL reverse transcribed cDNA in glass capillaries using a Light Cycler system (Roche Diagnostics), as previously described (25). Primer sequences were as follows: IP-10, sense 5′-TCCCTCTCGCAAGGAC-3′ and reverse 5′-TTGGCTAAACGCTTTCAT-3′; MIP-2, sense 5′-ATGAAGCTCTGCGTGT-3′ and reverse 5′-GGCTCACTGGGGTTAG-3′; GAPDH, sense 5′-ATCCCAGAGCTGAACG-3′ and reverse 5′-AAGT-CGCAGGAGACA-3′. The amplified product was detected by the presence of a SYBR green fluorescent signal. Melting curve analysis and gel electrophoresis was used to document the amplicon specificity. The crossing point (Cp) of the log-linear portion of the amplification curve was determined. The relative induction of gene mRNA expression was calculated using the following equation $E^{\Delta Cp\ (\text{control samples} - \text{treated samples})}$ and normalized for the expression of GAPDH. Samples from quercetin fed TNFΔARE/WT mice were measured as a fold of the control propylene glycol fed TNFΔARE/WT mice.

Western blot analysis. Mode-K cells were pretreated with quercetin and its bacterial products (100 μmol/L) for 1 h followed by the stimulation with TNF (5 μg/L) for 0–180 min. Cells were lysed in 1× Laemmli buffer and 20–50 μg of protein was subjected to electrophoresis on 10% SDS-polyacrylamide (SDS-PAGE) gels. Antiphospho-RelA (Ser536), antihistone 3, antiphospho-Akt (Ser473), Akt (all from Cell Signaling), anti-RelA and anti-IκBα were used to detect immunoreactive phospho-RelA, histone 3, phospho-Akt, Akt, RelA, and IκBα, respectively, using the ECL Western blotting chemiluminescence detection kit (Amersham) as previously described (30).

Histone acetyl transferase (HAT) assay. Mode-K cells were pretreated with quercetin and its bacterial metabolites (100 μmol/L) for 1 h followed by the stimulation with TNF (5 μg/L) for an additional 20 min. Nuclear extracts were prepared according to the manufacturer's instructions (Active Motif) using Tri(2-carboxyethyl) phosphine (TCEP) (Sigma-Aldrich) in the lysis buffer. Extracts (25 μg) were used to determine HAT activity using HAT Activity Colorimetric Assay Kit (BioVision). The absorbance was read at 440 nm using a MultiScan spectrophotometer.

ELISA analysis. Mode-K cells were pretreated with quercetin and bacterial products (100 μmol/L) for 1 h followed by the stimulation with TNF (5 μg/L) for an additional 24 h. Protein concentrations were determined in spent culture supernatants of IEC cultures. IP-10 and MIP-2 production was determined by mouse-specific ELISA kits, according to the manufacturer's instructions (R&D Systems).

Reporter (SEAP) gene assay for NF-κB transcriptional activity. Mode-K cells were grown to 80% confluency and then transfected with 2 μg of the NF-κB-inducible reporter plasmid pNiFty-SEAP (InvivoGen) in the presence of 6 μL FuGENE 6 Transfection Reagent (Roche Diagnostics). The pNiFty-SEAP reporter construct contains an engineered ELAM promoter with 5 NF-κB binding sites (GGGGACTTTCC) and the secreted alkaline phosphatase (SEAP) as reporter gene. Stable transfected cells were selected after the initial transfection (48 h) in the presence of the antibiotic zeocin (InvivoGen). pNiFty-SEAP transfected Mode-K cells were pretreated for 1 h with quercetin and bacterial metabolites (100 μmol/L) followed by the stimulation with TNF (5 μg/L) for an additional 24 h. The secreted SEAP was measured according to the manufacturer's instructions (InvivoGen) at 405 nm in a MultiScan spectrophotometer.

Chromatin immunoprecipitation (ChIP) analysis. After the treatment of Mode-K cells with quercetin and TNF, chromatin immunoprecipitation was performed using the ChIP-IT kit from Active Motif as described by the manufacturer. Extracts were normalized according to their DNA concentration, and immunoprecipitations were carried out using 5 μL antiphospho-RelA (Ser536), antiacetylated-phosphorylated H3 (Cell Signaling), and anti-CBP/p300 antibodies (Biomol). DNA isolated from an aliquot of the total nuclear extract was used as a loading control for the PCR (input control). PCR was performed with total DNA and immunoprecipitated DNA using the following IP-10 promoter-specific primers 5′-AACAGCTCACGCTTTG-3′, 5′-GTCCTGATTGGCTGACT-3′, and MIP-2 promoter-specific primers 5′-GCCTATCGCCAATGAGC-3′, 5′-CAATTTTCTGAACCAAGGG-3′. The length of the amplified product was 186 bp and 185 bp, respectively. The PCR products were subjected to electrophoresis on 2% agarose gels.

Statistical analysis. Values are expressed as means ± SD of 9 independent experiments. Differences were determined on log-transformed data using 1-way ANOVA followed by Tukey's test. Significance was set at $P < 0.05$.

Results

Quercetin inhibited TNF-induced IP-10 and MIP-2 expression. Quercetin, but none of its bacterial metabolites, significantly inhibited MIP-2 and completely blocked IP-10 protein secretion after TNF stimulation in Mode-K cells (**Table 1**). Dose-response analysis for quercetin-mediated IP-10 (**Fig. 2***A*) and MIP-2 (Fig. 2*B*) inhibition revealed effective concentrations of 40 and 44 μmol/L, respectively. Of note, quercetin did not induce significant cytotoxicity (<15%). Interestingly, taxifolin, alphitonin, and 3,4-dihydroxy-phenylacetic acid did not inhibit TNF-induced IP-10 and MIP-2 protein production, suggesting that bacterial transformation of quercetin during the intestinal transit may reduce the anti-inflammatory mechanism of this polyphenolic compound.

Quercetin did not inhibit NF-κB RelA phosphorylation and NF-κB reporter gene activity. Because the pharmacological NF-κB (PDTC) and PI3 kinase (LY294002) inhibitors blocked TNF-induced IP-10 and MIP-2 production (**Table 2**), we next investigated the effects of quercetin on TNF-induced Akt and RelA phosphorylation (S536) as well as IκBα degradation in Mode-K cells.

Quercetin almost completely inhibited Akt phosphorylation in TNF treated Mode-K cells after 20 min of stimulation (**Fig. 3**, lane 4). Consistent with lack of inhibitory functions on IP-10 and MIP-2 expression, the quercetin metabolites taxifolin, alphitonin, and 3,4-dihydroxy-phenylacetic acid did not affect the level of Akt phosphorylation (Fig. 3, lanes 5–7).

Time-response analysis revealed transient TNF-induced RelA phosphorylation after 20 min (**Fig. 4***A*, lane 3) followed by almost complete degradation of IκBα protein after 60 min of stimulation (Fig. 4*A*, lane 5). Based on the kinetic analysis of signal-specific protein phosphorylation/degradation, we next measured TNF-induced RelA phosphorylation (S536) after 20 min and IκBα degradation after 60 min of stimulation in the absence and presence of quercetin, taxifolin, alphitonin, and 3,4-dihydroxy-phenylacetic acid. Although quercetin blocked Akt phosphorylation as well as NF-κB–dependent IP-10 and MIP-2 expression, quercetin did not modulate TNF-induced

TABLE 1 Differential effects of quercetin on TNF-induced IP-10 and MIP-2 expression in Mode-K cells[1]

	IP-10	MIP-2
	μg/L	
CTRL	31.1 ± 3.3[a]	64.3 ± 21.4[a]
DMSO	20.3 ± 2.2[a]	88.1 ± 152.6[a]
TNF+DMSO	265.5 ± 23.0[b]	1828.6 ± 142.7[b]
TNF+quercetin	58.2 ± 9.8[a]	769.1 ± 39.3[a]
TNF+taxifolin	234.9 ± 33.3[b]	1354.8 ± 153.6[b]
TNF+alphitonin	321.4 ± 37.2[b]	1659.5 ± 325.3[b]
TNF+3,4-dihydroxy-phenylacetic acid	307.7 ± 39.5[b]	2019.1 ± 304.8[b]

[1] Values are means ± SD, $n = 9$. Means in a column with superscripts without a common letter differ, $P < 0.01$.

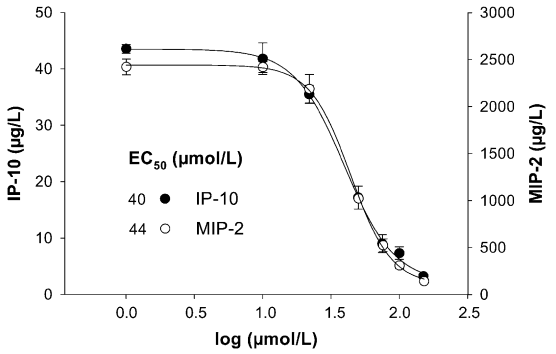

FIGURE 2 Effective inhibitory concentration of quercetin in Mode-K cells. TNF-induced IP-10 and MIP-2 protein concentrations were measured in the spent culture supernatant using ELISA and blotted against the flavonoid concentration to determine the effective inhibitory concentration. Medium alone and medium with vehicle (DMSO) were used as controls. The results are means ± SD of 2 independent experiments performed in duplicate 6-well cultures.

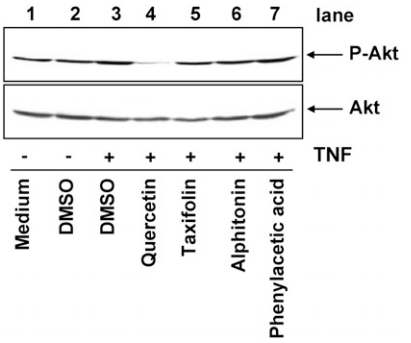

FIGURE 3 Quercetin inhibited Akt phosphorylation in Mode-K cells. Mode-K cells were stimulated with TNF in the presence of quercetin, taxifolin, alphitonin, and 3,4-dihydroxy-phenylacetic acid. Medium alone and medium with vehicle (DMSO) were use as controls. These results are representative of 2 independent experiments.

RelA phosphorylation (Fig. 4*B*, lane 4 vs. 3) as well as IκBα degradation (Fig. 4*B*, lane 4 vs. 3). Consistent with our previous results, taxifolin, alphitonin, and 3,4-dihydroxy-phenylacetic acid also did not affect TNF-induced NF-κB/IκBα activation (Fig. 4*B*, lanes 5–7).

Quercetin did not modulate NF-κB transcriptional activity after stimulation of pNiFty-SEAP transfected Mode-K cells with TNF, but significantly inhibited HAT activity, suggesting that quercetin may specifically affect chromatin remodeling at native gene promoters (**Table 3**). Taxifolin, alphitonin, and 3,4-dihydroxy-phenylacetic acid did not affect TNF-induced NF-κB reporter gene or HAT activity (Table 3).

Quercetin inhibited TNF-induced NF-κB and cofactor recruitment to the IP-10 and MIP-2 gene promoters. The treatment of Mode-K cells with quercetin inhibited TNF-induced phospho-RelA recruitment to the IP-10 (**Fig. 5***A*) and MIP-2 promoter, as shown by ChIP analysis (Fig. 5*B*). In addition, and consistent with the reduced HAT activity (Table 3), quercetin inhibited TNF-induced acetylation/phosphorylation of H3 and CBP/p300 binding at the IP-10 (Fig. 5*A*) and MIP-2 (Fig. 5*B*) gene promoters.

Quercetin inhibited IP-10 and MIP-2 gene expression in primary ileal IEC from inflamed TNFΔARE/WT mice. The histopathological analysis revealed moderate to severe inflammatory processes in the terminal ileum of quercetin-fed TNFΔARE/WT mice (histological score: 3.9 ± 0.7); this score did not differ from that of the propylene glycol fed control TNFΔARE/WT mice (histological score: 4.4 ± 0.6). WT mice did not show histopathological changes in the terminal ileum (histological score <1) (**Fig. 6***A*).

TABLE 2 Effect of inhibitors on TNF-induced IP-10 and MIP-2 expression in Mode-K cells[1]

	IP-10	MIP-2
	μg/L	
CTRL	0.4 ± 0.4[a]	138.1 ± 35.8[a]
PDTC	0.9 ± 0.1[a]	109.5 ± 74.3[a]
LY294002	1.3 ± 0.1[a]	66.7 ± 43.1[a]
SB203580	0.2 ± 0.1[a]	207.1 ± 25.8[a]
TNF	133.1 ± 8.4[b]	1488.1 ± 10.9[b]
TNF+PDTC	0.9 ± 0.2[a]	64.3 ± 12.4[a]
TNF+LY294002	0.2 ± 0.1[a]	142.9 ± 53.9[a]
TNF+SB203580	82.6 ± 17.0[b]	1342.9 ± 98.2[b]

[1] Values are means ± SD, $n = 9$. Means in a column with superscripts without a common letter differ, $P < 0.01$.

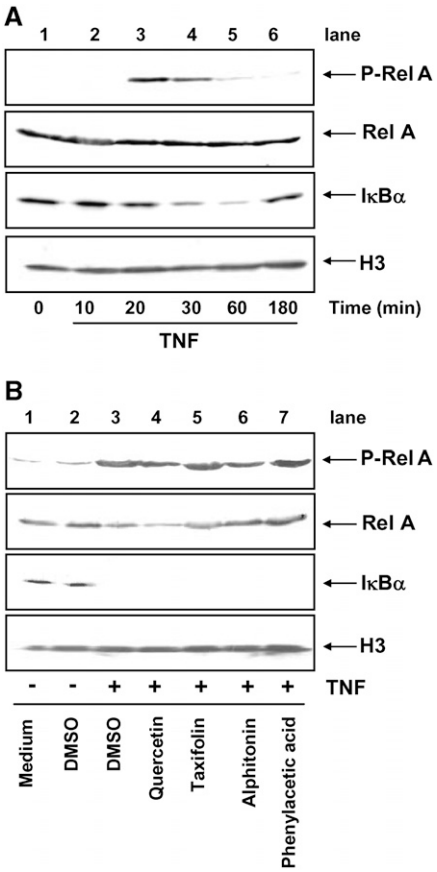

FIGURE 4 Quercetin did not inhibit TNF-induced NF-κB RelA phosphorylation and IκBα degradation in Mode-K cells. Kinetic analysis for the TNF-induced activation of the NF-κB/IκB complex (*A*). Mode-K cells were stimulated with TNF for 10, 20, 30, 60, and 180 min. Medium alone and medium with vehicle (DMSO) were use as controls. Mode-K cells were stimulated with TNF in the presence of quercetin, taxifolin, alphitonin and 3, 4-dihydroxy-phenylacetic acid (*B*). There results are representative of 2 independent experiments.

TABLE 3 Effect of quercetin and its metabolites on TNF-induced NF-κB reporter gene activity and histone acetyl transferase (HAT) activity in Mode-K cells[1]

	NF-κB reporter activity	HAT activity, *% of TNF+DMSO*
CTRL	1.0 ± 0.2[a]	
DMSO	0.8 ± 0.2[a]	
TNF+DMSO	6.0 ± 0.6[b]	100.0 ± 3.2[a]
TNF+quercetin	5.4 ± 0.2[b]	61.1 ± 7.1[b]
TNF+taxifolin	6.4 ± 0.7[b]	75.4 ± 4.1[a]
TNF+alphitonin	5.9 ± 0.4[b]	85.7 ± 9.7[a]
TNF+3,4-dihydroxy-phenylacetic acid	5.4 ± 0.9[b]	88.8 ± 2.7[a]

[1] Values are means ± SD, $n = 9$. Means in a column with superscripts without a common letter differ, $P < 0.05$.

Although quercetin feeding did not inhibit tissue inflammation, IP-10 and MIP-2 mRNA expression were significantly reduced in IEC from quercetin-fed TNFΔARE/WT mice compared with control TNFΔARE/WT mice (Fig. 6*B*). Consistent with the reduced IP-10 and MIP-2 gene expression in primary IEC, NF-κB nuclear staining was strongly reduced in quercetin-fed TNFΔARE mice (Fig. 6*C*).

Discussion

In this study, we showed that the polyphenolic plant-derived flavonoid quercetin inhibits TNF-induced expression of the proinflammatory cytokines IP-10 and MIP-2 in primary IEC from TNFΔARE/WT mice as well as the epithelial cell line Mode-K. Consistent with its inhibitory function on various protein kinases (8), quercetin inhibited Akt phosphorylation in Mode-K cells but did not inhibit TNF-induced NF-κB/IκB phosphorylation/degradation or NF-κB reporter gene activity. Interestingly, and most important for understanding the mechanism involved, quercetin inhibited the recruitment of the NF-κB cofactor CBP/p300 to the IP-10 and MIP-2 gene promoters, suggesting that quercetin may target the TNF-induced transcriptional regulation at the chromatin. Indeed, we demonstrated that quercetin reduced total HAT activity and blocked TNF-induced acetylation and/or phosphorylation of H3 at the IP-10 and MIP-2 gene promoters. It seems likely that the inhibitory effect of quercetin on the PI3 kinase/Akt signaling cascade may directly affect the NF-κB-dependent gene expression by modulating CBP/p300 recruitment and/or HAT activity at the chromatin (**Fig. 7**).

The transcriptional coactivators CBP and p300 play a central role in integrating various signal transduction pathways by coordinating the communication of transcription factors with the transcriptional apparatus to modulate stimulus-specific gene activity (47). Interestingly, CBP/p300 exerts its transcription-regulating properties by facilitating protein-protein interactions (47) as well as by its intrinsic HAT activity (48). The serine/threonine kinase Akt has been previously shown to interfere with the NF-κB signaling cascade at various levels, including the induction of IκB kinase (IKK)β activity, NF-κB DNA binding activity, and NF-κB transcriptional activity (30,49–51). It was recently demonstrated that TNF-induced nuclear Akt associates with CBP/p300 to phosphorylate the transcriptional coactivator at serine residue 1834 (52) and to repress CBP/p300 proteasomal degradation (53). The authors showed that p300 serine phosphorylation triggered histone acetylation and recruitment to the NF-κB-dependent gene promoter ICAM-1 (52). Quercetin did not block TNF-induced RelA phosphorylation and IκBα degradation, suggesting that the inhibition of the PI3-kinase/Akt pathway did not affect cytoplasmic IKK activity. Interestingly, Mayo et al. (54) showed in prostate cells that the reintroduction of PTEN, which is a lipid phosphatase responsible for the deactivation of PI3K/Akt signaling, results in the inhibition of TNF-induced NF-κB transcriptional activity by blocking the transactivation domain of the RelA/p65 subunit. Consistent with our findings, the authors showed that PTEN did not inhibit TNF-induced IKK activity, IκBα degradation, and NF-κB RelA nuclear translocation but blocked transcriptional activation of NF-κB specific genes. These results strongly suggest that the inhibition of Akt phosphorylation by quercetin subsequently blocked TNF-induced phospho-RelA and CBP/p300 nuclear

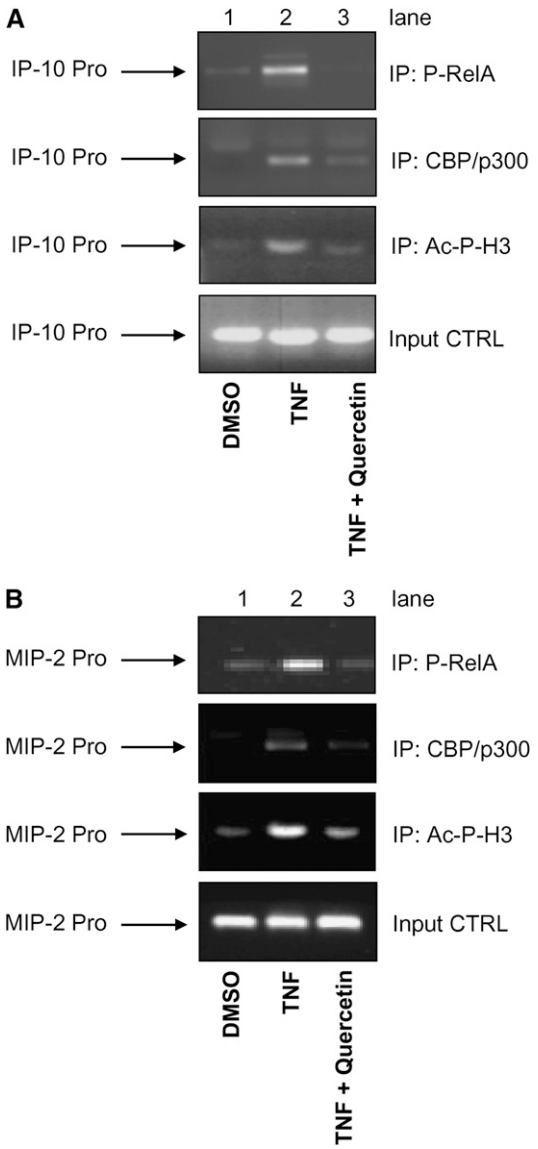

FIGURE 5 Quercetin inhibited TNF-induced NF-κB RelA and CBP/p300 binding to the IP-10 and MIP-2 gene promoters in Mode-K cells (*A,B*). Mode-K cells were stimulated with TNF in the absence and presence of quercetin. Medium with vehicle (DMSO) was used as control. ChIP analysis was performed using antiphospho-RelA, anti-CBP/p300, and antiacetylated/phosphorylated H3 antibodies for immunoprecipitation followed by IP-10 (*A*) and MIP-2 (*B*) promoter specific PCR. These results are representative of 3 independent experiments.

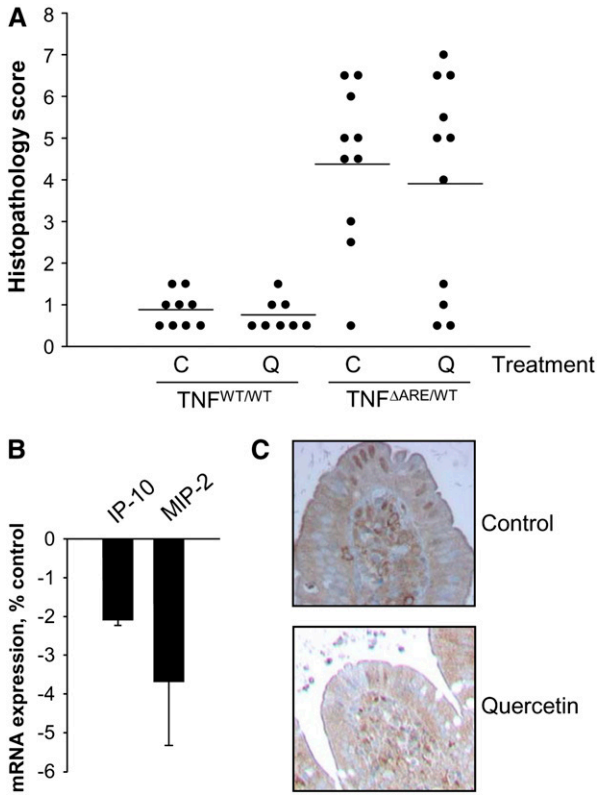

FIGURE 6 Quercetin inhibited IP-10 and MIP-2 gene expression in primary ileal epithelial cells but did not modulate tissue pathology in heterozygous TNFΔARE/WT mice and WT mice fed quercetin (Q) or propylene glycol (C) for 10 wk. Values are individual mice (*A*) or means ± SD, $n = 8$–11 (*B*). Panel *C* shows representative nuclear RelA staining.

recruitment at the IP-10 and MIP-2 gene promoters through the modulation of histone 3 acetylation/phosphorylation.

Despite the significantly reduced NF-κB nuclear staining, as well as IP-10 and MIP-2 mRNA expression in native IEC from quercetin-treated TNFΔARE/WT, these mice showed no differences in the severity of mucosal inflammation, indicating that the inhibition of IEC activation may not be sufficient to compensate for the pathologic mechanisms of TNF over-production in deeper layers of the mucosa. Recently, Comalada et al. (15) showed that aglycon quercetin, but not its glycoside form, quercitrin (3-rhamnosyl-quercetin), inhibited macrophage activation/proliferation as well as iNOS expression. In contrast, only oral administration of quercitrin was able to inhibit dextran sodium sulfate-induced colitis (15). This finding was further supported by the fact that fecal bacteria triggered the release of quercetin from its glycoside quercitrin. In addition, quercitrin showed protective effects in the intestinal epithelium of TNBS-treated mice but did not inhibit tissue inflammation (20).

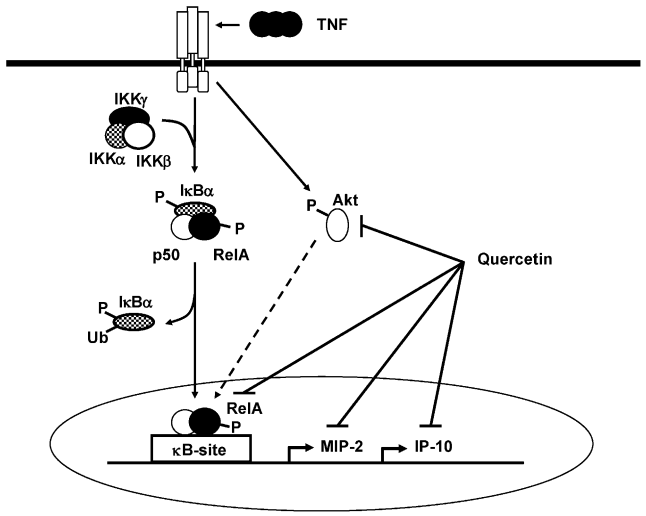

FIGURE 7 Schematic illustration for the inhibitory mechanism of quercetin. Inhibition of Akt phosphorylation was associated with the inhibition of TNF-induced recruitment of phospho-RelA to the pro-inflammatory gene promoters, abrogating recruitment of CBP/p300 binding and histone acetylation/phosphorylation.

These results clearly suggest that the mechanistically active anti-inflammatory compound, quercetin, was generated after cleavage of the glycoside residue during colonic transit. Additional studies showed that the oral application of quercitrin to trinitrobenzene sulfonic acid (TNBS)-treated rats normalized hydroelectrolytic fluid transport in the intestinal epithelum but did not inhibit the development of TNBS-induced histopathology and myeloperoxidase activity in the colonic mucosa, suggesting that the protective effects of quercetin may vary among the various animal models of experimental colitis (20).

It seems important to understand that a major part of the ingested flavonoids are not metabolized by the gut. Several studies concerning the absorption and bioavailability of quercetin in humans show that although the absorption of quercetin aglycone has been reported to be ~24%, the absorption of quercetin glycosides from onions was 52% (2,55). The dietary source of quercetin has also been reported to play an important role in the final concentration of this flavonol in plasma. Thus, the bioavailability of quercetin from both apples and pure quercetin rutinoside was only 30% compared with onions (56), with decreasing values when ingested from black tea and wine (57,58). Flavonoids and their glycosides are partially degraded by strictly anaerobic colonic bacteria including the species *Clostridium scindens*, *Clostridium orbiscindens*, *Eubacterium desmolans* and *Eubacterium ramulus* (59,60). For example, *Eubacterium ramulus* is a flavonoid-degrading member of the normal flora whose unique carbon and energy source is quercetin-3-glucoside (isoquercetin) (42). The degradation of quercetin by these Gram-positive anaerobic bacteria results in the metabolite products taxifolin, alphitonin, 3,4-dihydroxy-phenylacetic acid, and phloroglucinol (43). Because we demonstrated that the quercetin metabolites taxifolin, alphitonin, and 3, 4-dihydroxy-phenylacetic acid and did not inhibit TNF-induced proinflammatory gene expression in IEC, we sought to measure the effects of quercetin in the chronically inflamed small intestine using heterozygous TNFΔARE/WT mice as an animal model of experimental ileitis. Interestingly, the oral application of quercetin did not modulate the overall tissue damage in the ileum under conditions of chronic inflammation. On the other hand, the expression of the proinflammatory cytokines IP-10 and MIP-2 were significantly inhibited in primary ileal epithelial cells from quercetin-treated TNFΔARE/WT mice compared with the propylene glycol control group, supporting, at least to some extent, our mechanistic in vitro studies on the inhibitory effect of quercetin on TNF-induced IEC activation.

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