Chrysin Ameliorates Chemically Induced Colitis in the Mouse through Modulation of a PXR/NF- κ B Signaling Pathway

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ABSTRACT

Targeted activation of pregnane X receptor (PXR) in recent years has become a therapeutic strategy for inflammatory bowel disease. Chrysin is a naturally occurring flavonoid with anti-inflammation activity. The current study investigated the role of chrysin as a putative mouse PXR agonist in preventing experimental colitis. Pre-administration of chrysin ameliorated inflammatory symptoms in mouse models of colitis (dextran sodium sulfate- and 2,4,6-trinitrobenzene sulfonic acid-induced) and resulted in down-regulation of nuclear transcription factor κ B (NF- κ B) target genes (inducible NO synthase, intercellular adhesion molecule-1, monocyte chemotactic protein-1, cyclooxygenase 2, tumor necrosis factor- α , and interleukin 6) in the colon mucosa. Chrysin inhibited the phosphorylation/degradation of inhibitor κ B α (I κ B α), which correlated with the decrease in the activity of myeloperoxidase and the levels of tumor necrosis

factor- α and interleukin 6 in the colon. Consistent with the *in vivo* results, chrysin blocked lipopolysaccharide-stimulated nuclear translocation of NF- κ B p65 in mouse macrophage RAW264.7. Furthermore, chrysin dose-dependently activated human/mouse PXR in reporter gene assays and up-regulated xenobiotic detoxification genes in the colon mucosa, but not in the liver. Silencing of PXR by RNA interference demonstrated necessity of PXR in mediating chrysin's ability to induce xenobiotic detoxification genes and NF- κ B inactivation. The repression of NF- κ B transcription activity by chrysin was confirmed by *in vitro* PXR transduction. These findings suggest that the effect of chrysin in preventing chemically induced colitis is mediated in large part by a PXR/NF- κ B pathway. The data also suggest that chrysin or chrysin-like flavonoids could be further developed as intestine-specific PXR activators.

Introduction

Pregnane x receptor (PXR), a ligand-activated transcription factor and master regulator of xenobiotic metabolism and intestinal immune homeostasis, is abundantly expressed in the intestine and liver of mammals and rodents (Fiorucci et al., 2012). More recently, PXR has been implicated in the pathogenesis of inflammatory bowel disease (IBD), a chronic inflammation of the intestinal tract (Langmann et al., 2004; Cheng et al., 2010). After activation, PXR behaves as a xenosensor and regulates a battery of genes encoding metabolism enzymes [e.g., cytochromes P450 (P450s), UDP-glucuronosyltransferases (UGTs),

and glutathione S-transferase] and xenobiotic transporters [e.g., multidrug resistance 1 (MDR1), multidrug resistance protein 2, and organic anion transporting polypeptide 2]. Attenuated activation of PXR target genes, such as the xenobiotic transporters MDR1 (ABCB1), leads to a less compact epithelial barrier (Ma et al., 2007). Therefore, impaired PXR function may lead to less effective induction of MDR1 and export of harmful substances that originate from bacteria, diet, and pollutants (Langmann et al., 2004; Elias and Mills 2007). For instance, there was a significant down-regulation of PXR and reduced expression of drug metabolism enzymes and xenobiotic transporters in the colon of patients with IBD, suggesting a role of PXR and its target genes in the pathogenesis of IBD (Blokzijl et al., 2007; Mencarelli et al., 2010). In addition, PXR was shown to be a key regulator of inflammation in hepatocytes and in the intestine. PXR activation inhibited the activity of nuclear transcription factor κ B (NF- κ B) and the expression of its target genes (Shah et al., 2007). Recent studies have demonstrated that IBD therapeutic drugs that typically activate PXR, such as

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ABBREVIATIONS: Cox2, cyclooxygenase 2; DSS, dextran sodium sulfate; IBD, inflammatory bowel disease; ICAM-1, intercellular adhesion molecule-1; IL-6, interleukin 6; iNOS, inducible NO synthase; I κ B α , inhibitor κ B α ; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; MDR1, multidrug resistance 1; MPO, myeloperoxidase; MW, molecular weight; NF- κ B, nuclear transcription factor κ B; P450, cytochrome P450; PBS, phosphate-buffered saline; PXR, pregnane x receptor; qRT-PCR, reverse transcription quantitative real-time polymerase chain reaction; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNF- α , tumor necrosis factor- α ; UGTs, UDP-glucuronosyltransferases.

rifaximin, inhibited NF- κ B and its target genes, such as inducible NO synthase (iNOS), intercellular adhesion molecule-1 (ICAM-1), chemokine receptor 2, monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor- α (TNF- α), and several interleukins (ILs) (Ma et al., 2007; Cheng et al., 2010). Our previous study indicated that natural flavonoid baicalein ameliorated dextran sodium sulfate (DSS)-induced colitis via a caudal-related homeodomain transcription factor 2-mediated PXR activation mechanism (Dou et al., 2012).

Chrysin (5,7-dihydroxyflavone), a naturally occurring flavonoid in many plant extracts, honey, and propolis, has been used as a traditional anti-inflammatory and anti-cancer remedy for centuries (Woo et al., 2005). A recent investigation suggested that chrysin coadministration ameliorated inflammatory symptoms in a mouse model of DSS-induced colitis and blocked NF- κ B activation in intestinal epithelial IEC-6 cells (Shin et al., 2009). In the past, chrysin was demonstrated to be a human PXR ligand in vitro (Sugatani et al., 2004). However, the ability of chrysin to activate PXR in vivo and its resulting phenotype remain unknown. On the basis of the key role that PXR plays in intestinal inflammation, we investigated the effect of chrysin in PXR activation and evaluated PXR/NF- κ B signaling pathway in ameliorating DSS- and trinitrobenzene sulfonic acid (TNBS)-induced colitis.

Materials and Methods

Materials. Chrysin (5,7-dihydroxyflavone, C₁₅H₁₀O₄, molecular weight (MW) 254.24, high-performance liquid chromatography $\geq 98\%$) was obtained from Shanghai R&D Center for Standardization of Traditional Chinese Medicine (Shanghai, China). Mouse macrophage RAW264.7 cells, human colorectal carcinoma HT-29 cells, and human hepatocyte carcinoma HepG2 cells were purchased from ATCC (Manassas, VA). All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum under 5% CO₂ at 37°C. For in vivo studies, chrysin stock solution was prepared in 0.5% methylcellulose and administered to mice at a dose of 25 mg/kg/day by oral gavage. As vehicle control, all mice received an equivalent volume (compared with experimental groups) of 0.5% methylcellulose solution. Healthy 8-week-old female C57BL/6 mice (weight, 20 $\pm$ 2 g) were obtained from Shanghai Laboratory Animal Center, and studies were performed in accordance with the guidelines approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine. All mice were housed under a specific pathogen-free facility at the university with free access to standard laboratory chow and tap water.

DSS-Induced Colitis. Eight-week-old female C57BL/6 mice were placed into four groups (≥ 10 per group) in the DSS-induced IBD study. In group 1, vehicle controls were administered 100 μ l of 0.5% methylcellulose by oral gavage once per day. In group 2, chrysin at a dose of 25 mg/kg of body weight via oral gavage was given once per day. In group 3, 100 μ l of 0.5% methylcellulose by oral gavage was given once per day, and 4% DSS (MW 36000–50000; MP Biomedicals, Solon, OH) in drinking water was given on days 4–10. Group 4 received chrysin by oral gavage (25 mg/kg of body weight) 3 days before DSS treatment and continued to the end of DSS treatment.

TNBS-Induced Colitis. Eight-week-old female C57BL/6 mice were placed into four groups (10 per group) in the TNBS-induced IBD study. For group 1 and group 2, refer to the DSS-induced IBD model. In group 3, 100 μ l of 0.5% methylcellulose was given by oral gavage once per day, and 2 mg (in 100 μ l of 50% ethanol) of TNBS (Sigma-Aldrich, St. Louis, MO) was administered intrarectally to fasted and anesthetized mice via a catheter inserted 3 cm proximally

to the anus on day 4. Group 4 received chrysin by oral gavage (25 mg/kg of body weight) 3 days before TNBS administration and continued to the end of the study (day 10).

Colitis Evaluation. Mice were monitored daily for the signs of body movement, weight, diarrhea, and bloody stool. Bloody diarrhea events were evaluated clinically by inspection of anal discharge, and a percentage value was determined on the basis of the number of animals with this condition at any given time (Wallace et al., 2010). Animals were sacrificed by cervical dislocation under anesthesia. The entire colon was excised and placed on the ice plate and cleaned of fat and mesentery. The length of each colon specimen was measured. The distal colons were taken and fixed in 10% buffered formalin for 24 hours at room temperature, embedded in paraffin, and stained with H&E for histologic evaluation. Histologic scoring was performed in a blinded fashion by two pathologists (Liangwen Gu and Mengqing Han) as a combined score of inflammatory cell infiltration (score, 0–3) and tissue damage (score, 0–3), as described previously (Siegmund et al., 2001; Wallace et al., 2010; Dou et al., 2012). The two subscores were added, and the combined histologic score ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage).

Western Blot Analysis. Colon tissues were disrupted by homogenization on ice and centrifuged at 4°C (12,000g, 15 minutes). The supernatants were collected, and protein concentrations were determined. Equal amounts of protein (40 μ g/lane) were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked in 5% skim milk and incubated with antibodies against PXR (ab85451; Abcam, Cambridge, MA), phospho-p65 (#3033, 1:1000; Cell Signaling Technology Inc., Beverly, MA), phospho-I κ B α (#2859, 1:1000; Cell Signaling), I κ B α (#4812, 1:1000; Cell Signaling), and β -actin (#4970; 1:2000; Cell Signaling). Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were developed by ECL detection reagents (GE Healthcare, Waukesha, WI). The protein bands were quantified using the mean ratios of integral optic density after normalization to β -actin expression.

RNA Analysis. RNA was extracted from colon and liver samples with use of TRIzol reagent (Invitrogen, Carlsbad, CA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using cDNA generated from 3 μ g total RNA with the SuperScript II Reverse Transcriptase kit (Invitrogen). The primer sequences used in PCR amplification are as follows: 5'-GATGAAAGAAAGTCGCCTCG-3'/5'-GCTGGACATCAGGGTGAGTG-3' for hCyp3a4, 5'-AGCCCATCCTGTTTACTGTC-3'/5'-TGTATGTTGGCCTCCTTTGTC-3' for hMdr1, 5'-GGAAATCGTGCCTGACATTA-3'/5'-TCAGGCAGCTCGTAGCTCTT-3' for h β -actin, 5'-TGGAGATGGAATACCTGGAT-3'/5'-GAATCATCACTGTTGACCT-3' for mCyp3a11, 5'-TGTGATTGCGTTTGGAGGAC-3'/5'-CCATACCAGAATGCCAGAGC-3' for mMdr1a, 5'-GGGAATCTTGGAGCGAGTTG-3'/5'-GTGAGGGCTTGGCTGAGTGA-3' for mI κ B, 5'-CGCTGTGCTTTGAGAACTGT-3'/5'-AGGTCCTTGCC-TACTTGCTG-3' for mICAM-1, 5'-AAGTTGACCCGTAAATCTGA-3'/5'-TGAAAGGGAATACCATAACA-3' for mMCP-1, 5'-GAAGTCTTTG-TCTGTGGTGCCCT-3'/5'-GCTCCTGCTTGAGTATGTCG-3' for mCox2 (cyclooxygenase 2), 5'-ACCACGGCCTTCCCTACTTC-3'/5'-CATTTCCACGATTTCCAGA-3' for mIL-6 (interleukin 6), 5'-CGTGGAAGTGGCAGAAAGAGG-3'/5'-AGACAGAAGAGCGTGGTGCC-3' for mTNF- α , and 5'-CAGCCTTCTCTTCTGGGTAT-3'/5'-TGGCATAGAGGTCTTTACGG-3' for m β -actin. PCR was performed using SYBR Premix ExTaq Mix (Takara Biotechnology, Otsu, Japan) in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Thermal cycler parameters were as follows: 1 cycle of 95°C for 30 seconds, 40 cycles of denaturation (95°C, 5 seconds), and combined annealing/extension (60°C, 30 seconds). Gene expression changes were calculated by the comparative Ct method, and the values were normalized to endogenous reference β -actin.

Determination of TNF- α and IL-6 Levels. Colon segments were homogenized in ice-cold phosphate-buffered saline (PBS). The homogenates were centrifuged at 3000g for 10 minutes, and the supernatants were assayed for the determination of levels of the

cytokines. The level of each cytokine was evaluated using enzyme-linked immunosorbent assay kits according to the manufacturer's protocols (R&D Systems, Minneapolis, MN), and the results are expressed in pg/mg of protein in each sample.

Determination of Myeloperoxidase Activity. Tissue myeloperoxidase (MPO) activity, which is linearly related to neutrophil infiltration in inflamed tissue, was assayed to monitor the degree of inflammation. MPO activity was measured in pieces of colon adjacent to the instillation point according to manufacturer's instructions (CytoStore, Calgary, AB, Canada). MPO activity is expressed as units/mg of protein.

NF- κ B Nuclear Translocation Immunofluorescence. RAW264.7 cells were seeded in 8-chamber slides (BD Biosciences, Bedford, MA) at a density of 5×10^4 cells per well. Cells were allowed to adhere at 37°C overnight, and chrysin (50 μ M) was added to the cells for 2 hours. Then, cells were stimulated overnight with lipopolysaccharide (LPS, 2 μ g/ml; Sigma-Aldrich) and fixed with 4% paraformaldehyde solution at 20°C for 10 minutes. After washing in PBS, cells were permeabilized with 0.3% Triton X-100 in PBS at room temperature for 20 minutes. After incubation in blocking buffer of 0.1% Triton X-100, 1% bovine serum albumin, and 3% donkey serum, cells were incubated with rabbit NF- κ B p65 antibody (#8242, 1:50; Cell Signaling) at 4°C overnight and further incubated with Alexa Fluor 488-conjugated anti-rabbit IgG (A-21206, 1:500; Invitrogen) at room temperature for 45 minutes; 1 μ g/ml of Dapi (Invitrogen) in PBS was added to stain the nuclei. Fluorescence photographs were obtained using an Olympus CKX41 fluorescence microscope.

PXR Transactivation Reporter Assay. HT-29 cells were maintained and transiently transfected using Lonza Nucleofector II instrument (Amaxa Biosystems, Lonza, Germany) as previously described (Dou et al., 2012). Receptor constructs contained coding sequences for human PXR (pSG5-hPXR) and mouse PXR (pCMV-mPXR) (Dou et al., 2012). The reporter used was CYP3A4-luciferase (pGL3-CYP3A4-Luc[(-444/+53)(-7836/-7208)]; Genscript Corp., Piscataway, NJ). The receptors were transfected in combination with the reporter. After electroporation, cells were incubated with chrysin (1.56, 3.12, 6.25, 12.5, 25, 50, 75, 100, and 125 μ M). Cell lysates were assayed for luciferase activity after compound treatment for 24 hours. Results were expressed as fold induction of control cells.

Chrysin on PXR-Mediated NF- κ B Repression Reporter Assay. A total of 1×10^6 HT-29 cells were electroporated with pGL4.32[luc2P/NF- κ B-RE/Hygro] reporter (Promega, Madison, WI) alone or coelectroporated with pCMV-mPXR and pRL-TK with use of a Lonza Nucleofector II instrument. The pGL4.32 reporter is an NF- κ B reporter vector containing NF- κ B response elements and firefly luciferase gene. After overnight incubation, cells were incubated with chrysin (0, 1, 50, and 100 μ M) for 2 hours, followed by an additional incubation with or without TNF- α (20 ng/ml; Cell Signaling) for 5 hours. A standard dual luciferase assay was performed with cell lysates as described, and results were expressed as fold induction of control cells (Dou et al., 2012).

PXR Silencing. A total of 1×10^6 HepG2 cells were electroporated with PXR siRNA (h) (sc-44057; Santa Cruz Biotechnology) targeting the human PXR mRNA. Control siRNA (sc-37007; Santa Cruz Biotechnology), a nontargeting siRNA, was used as a negative control. After transfection, cells were incubated with chrysin at a 50 μ M final concentration for 2 hours, followed by an additional incubation with or without TNF- α (20 ng/ml; Cell Signaling) for 5 hours. At the end of the incubation, cells were rinsed, scraped, and used in qRT-PCR or Western blot studies as described above.

Statistical Analysis. The data were analyzed using SPSS, version 16.0, statistical package (SPSS). Multiple comparisons were performed using one-way analysis of variance followed by least significant difference (LSD) *t* test. A value of $P < 0.05$ was considered to be statistically significant, and all results are presented as mean $\pm$ S.D.

Results

Chrysin Administration Attenuated DSS-Induced Colitis. A previous study demonstrated the therapeutic effect of chrysin on DSS-induced IBD (Shin et al., 2009). To assess whether chrysin would also provide protection to the same IBD model, we added dextran sulfate sodium (MW 36000–50000; MP Biomedicals) to the drinking water to induce the acute colitis in mice. The inflammation was mainly localized to the colon, with features resembling human ulcerative colitis. There was no weight loss observed in mice receiving vehicle or chrysin alone (groups 1 and 2, respectively). The body weight of mice in group 3 dramatically decreased from day 3 onward after DSS treatment. The mice receiving both DSS and chrysin (group 4) exhibited less significant weight loss than did the group 3 mice (Fig. 1A). Diarrhea symptoms appeared on or shortly after day 3. From day 3 to day 7, all the mice receiving DSS treatment only (group 3) experienced both diarrhea and bloody diarrhea, whereas none of the mice receiving vehicle or chrysin alone (groups 1 and 2, respectively) exhibited diarrhea at any point during the study. By the end of IBD study, all the group 3 mice were euthanized under anesthesia because of critical weight loss and bloody diarrhea. In contrast, the mice receiving both DSS and chrysin (group 4), exhibited less diarrhea and bloody diarrhea than did the group 3 mice (Fig. 1B). Colon shortening is an indirect marker of colonic inflammation (Okayasu et al., 1990). After 7 days of treatment with DSS in drinking water, there was a significant shortening of the colon length of mice in group 3, compared with the mice receiving both DSS and chrysin. This indicates that the oral administration of chrysin significantly ameliorated the symptom of colon shortening (Fig. 1C). Examination and scoring of colonic samples from each group established that chrysin protected mucosal epithelium from DSS-induced damage (Fig. 1, D and E). Mice treated with vehicle or chrysin alone exhibited intact crypt-villus structures and epithelial layer. DSS administration resulted in a paucity of intact crypt-villus structures and large inflammatory exudates across the thickness of the bowel wall. By contrast, chrysin administration to DSS-exposed mice resulted in significant protection of the colon crypt structures and less severe histologic inflammation.

Direct Inhibition of NF- κ B Activity In Vivo and In Vitro. NF- κ B is the key regulator of the immune and inflammatory responses (Zhou et al., 2006). As a transcription factor, NF- κ B controls an array of pro-inflammatory genes involved in inflammatory signaling cascade (Zhang et al., 2009). We hypothesized that the anti-inflammatory effect of chrysin in response to DSS-induced colitis correlated with the blockade of NF- κ B activation. A significant increase in the protein expression of phospho-p65 was observed in colonic tissues of DSS-induced model group (Fig. 2, A and B). Furthermore, the degradation and phosphorylation of I κ B α was induced in colonic tissues of DSS-exposed mice, compared with mice control. By contrast, administration of chrysin reduced phospho-p65 expression in DSS-induced colitis, and the phosphorylation/degradation of I κ B α was effectively suppressed. Consistent with the in vivo data, chrysin (50 μ M) inhibited NF- κ B p65 nuclear translocation, as indicated in LPS-stimulated mouse macrophage RAW264.7 cells (Fig. 2C). These results indicate that chrysin significantly inhibited NF- κ B activity in DSS-induced colitis by suppressing I κ B α

Fig. 1. Protective role of chrysin against DSS-induced colitis in mice. (A) Body weight changes after DSS induction of colitis. Data are plotted as percentage of basal body weight. (B) The occurrence of bloody diarrhea. Mice were evaluated for the occurrence of bloody diarrhea after DSS administration. Data are plotted as percentage of total mice on different point of time of DSS treatment. (C) Colon length. (D) Representative H&E-stained colon sections (magnification $\times 200$). (E) Histology score. DSS-induced IBD studies were repeated twice. Values were expressed as mean $\pm$ S.D. of 20 mice in each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, versus DSS-treated group.

phosphorylation/degradation and blocking NF- κ B p65 nuclear translocation.

Chrysin Activated Human/Mouse PXR and Up-Regulated PXR Target Genes in the Colon. A previous study indicated that chrysin activates human PXR and induces UGT1A1 promoter in a cell-based reporter assay (Shin et al., 2009). We hypothesized that chrysin could also activate mouse PXR and induce Cyp3a11 promoter. A transient transfection luciferase reporter assay was performed in colonic HT-29 cells to evaluate the effect of chrysin on human PXR- and mouse PXR-mediated CYP3A4 transcription activity, respectively. As

expected, chrysin activated both human and mouse PXR and induced CYP3A4 promoter activity in a dose-dependent manner (Fig. 3A). In addition, cell viability results showed less significant cytotoxicity of chrysin on the growth of transiently transfected HT-29 cells (Fig. 3B). Consistent with PXR-transactivation results, mRNA expression of mouse PXR target genes Cyp3a11 and Mdr1 was remarkably up-regulated in the colon, but not in the liver of mice exposed to chrysin (Fig. 3, C and D). These results indicate that chrysin is an activator of both human and mouse PXR and activates PXR in an intestine-specific manner.

Fig. 2. The effects of chrysin on NF- κ B repression in vivo and in vitro. (A) Mice (6 per group) were sacrificed after 7 days of 4% DSS exposure, and total protein (40 μ g) from colon samples was loaded. Western blot was performed with phospho-p65 (#3033, 1:1000), phospho-I κ B α (#2859, 1:1000), and I κ B α (#4812, 1:1000) antibodies. One representative experiment from three independent experiments is shown. (B) Quantification of the phospho-p65, phospho-I κ B α , and I κ B α protein expressions was performed by densitometric analysis of the blots. Data were expressed as the mean $\pm$ S.D. of three independent experiments. (C) RAW264.7 cells were pretreated with or without chrysin (50 μ M) for 2 hours, followed by an additional treatment by LPS (2 μ g/ml) for 12 hours. NF- κ B p65 localization was observed under a fluorescence microscope (magnification $\times$ 200) with use of an NF- κ B p65 antibody (#8242, 1:50) followed by an Alexa 488-conjugated detection antibody (A-21206, 1:500; n = 3). * P < 0.05; *** P < 0.001, versus DSS-treated group.

Inhibition of NF- κ B by Chrysin Was Mediated by Mouse PXR Expression. To determine the role of mouse PXR on the repression of NF- κ B signaling by chrysin, HT-29 cells were electroporated with a NF- κ B-luciferase reporter alone or coelectroporated with pCMV-mPXR and pRL-TK. After transfection, cells were incubated with vehicle or chrysin (1, 50, and 100 μ M) for 2 hours, followed by an additional incubation with or without TNF- α (20 ng/ml; Cell Signaling) for 5 hours. In cells transfected with NF- κ B reporter alone, TNF- α significantly induced NF- κ B luciferase expression, whereas chrysin had no effect on basal NF- κ B luciferase, and coincubation of chrysin (100 μ M) significantly repressed TNF- α -stimulated NF- κ B luciferase expression, compared with the cells treated with TNF- α alone (Fig. 3E). Compared with cells transfected with NF- κ B reporter alone, TNF- α -stimulated NF- κ B luciferase expression was significantly repressed after cotransfection with pCMV-mPXR, confirming the direct role of mouse PXR expression on NF- κ B inhibition. Furthermore, the addition of chrysin (50 and 100 μ M) potentiated the repression in a dose-dependent manner (Fig. 3E). We hypothesized that the significant inhibition of TNF- α -stimulated NF- κ B luciferase expression during chrysin (100 μ M) treatment in the absence of pCMV-mPXR transfection might be caused by basal expression of human PXR in HT-29 cells, because chrysin was also demonstrated to be a human PXR activator in the current study (human PXR transactivation assay). These results suggest that PXR-mediated NF- κ B suppression might be the mechanism by which chrysin provides protection to DSS-induced IBD.

Chrysin Inhibited NF- κ B Target Genes Expression in the Colon. NF- κ B is the central transcription factor in the regulation of pro-inflammatory cytokines and chemokines. To

elucidate the impact of chrysin on NF- κ B signaling, we investigated the expression levels of representative downstream signaling genes involved in NF- κ B activation. qRT-PCR analyses of several NF- κ B target genes were performed. The results showed that mRNA expression of iNOS, ICAM-1, MCP-1, Cox2, TNF- α , and IL-6 was remarkably induced in inflamed colons of mice exposed to DSS (group 3). By contrast, the increase in inflammatory mediators after DSS treatment was significantly decreased in mice receiving chrysin administration (Fig. 4, A–F). The results indicate that chrysin ameliorated DSS-induced colitis through NF- κ B signaling repression.

Effects of Chrysin on Detoxification Genes and NF- κ B Activity Were Abrogated by PXR Silencing. Previous studies have shown that inflammation-driven NF- κ B activation reduces PXR signaling and decreases hepatic P450 expression, whereas PXR activation inhibited the activity of NF- κ B and the expression of its target genes (Zhou, et al., 2006; Shah et al., 2007). To explore the mechanistic involvement of PXR in the effect exerted by chrysin, PXR gene expression was silenced by anti-PXR siRNA. We used human hepatocyte carcinoma HepG2 cells to assess the effects of chrysin caused by the abundant expression of PXR and its target genes in HepG2 cells. As shown in Fig. 5A, treatment of HepG2 cells with an anti-PXR siRNA markedly abrogated the expression of PXR. Exposure of HepG2 cells to TNF- α caused a significant increase in the protein expression of phospho-p65 and a significant degradation of I κ B α protein, and these effects were prevented by cotreatment with chrysin (Fig. 5, A and B). In addition, a robust reduction in the mRNA expressions of Cyp3a4 and Mdr1 was observed in HepG2 cells exposed to TNF- α ; this effect was reverted by cotreatment with chrysin (Fig. 5, C and D). Thus, chrysin effectively

Fig. 3. The effects for chrysin in human/mouse PXR activation, mouse PXR target gene expression, and mouse PXR-mediated NF- κ B repression. HT-29 cells were coelectroporated with CYP3A4 luciferase reporter and human or mouse receptor, respectively, as described in *Materials and Methods*. PXR transactivation assay (A) and cell viability assay (B) were performed to determine the effects for chrysin in the activation of human/mouse PXR and in cell viability, respectively. The results were presented as relative light unit (RLU), and normalization was performed. Data were expressed as mean $\pm$ S.D. of quadruplicates of three independent experiments. mRNA expressions of Cyp3a11 (C) and Mdr1 (D) were assessed by qRT-PCR in colon and liver samples isolated from mice (6 per group) treated with vehicle or chrysin (25 mg/kg gavage). Expression was normalized to β -actin, and each bar represents the mean $\pm$ S.D. of triplicates of two independent experiments. *** P < 0.001, versus vehicle control group. (E) Chrysin (Chry) on PXR-mediated NF- κ B repression reporter assay. HT-29 cells were electroporated with NF- κ B luciferase (LUC) reporter alone or coelectroporated with mouse PXR expression vector and along with pRL-TK. After transfection, cells were incubated with chrysin for 2 hours, followed by an additional incubation with or without TNF- α (20 ng/ml). A standard dual luciferase assay was performed with cell extracts, and results were expressed as mean $\pm$ S.D. of triplicates of three independent experiments. # P < 0.05 versus TNF- α -treated samples without mouse PXR transfection; * P < 0.05; ** P < 0.01, versus TNF- α -treated samples with mouse PXR transfection. Veh, vehicle.

enhanced the expression of PXR target genes and decreased the activity of NF- κ B in HepG2 cells exposed to TNF- α . However, silencing the expression of PXR by an anti-PXR siRNA almost completely abrogated the ability of chrysin to counteract the effects of TNF- α (Fig. 5, A–D). These results indicate that the effects of chrysin in the up-regulation of PXR target genes and in the suppression of NF- κ B activity were mediated by PXR expression.

Chrysin Reduced the Production of TNF- α and IL-6 in the Inflamed Colon. A significant increase in the content of TNF- α and IL-6 was observed in mice exposed to DSS (group 3), compared with control mice (group 1 and group 2). Treatment with chrysin resulted in a reduction in the levels of TNF- α and IL-6 in mice exposed to DSS (group 4), compared with mice receiving DSS alone (group 3) (Table 1). The data indicate that the protective effect for chrysin in DSS-induced colitis was correlated with the repression of pro-inflammatory cytokines.

Chrysin Decreased the Activity of MPO in the Inflamed Colon. MPO activity, a marker for leukocyte infiltration into the inflamed tissue, was low in the colonic tissues of control mice (group 1 and group 2) and markedly

(P < 0.001) increased in mice with DSS-induced colitis (Table 1). These results confirmed the histologic assessment, which showed increased leukocyte infiltration in mice with DSS-induced colitis. The increased MPO activity in mice with DSS-induced colitis was significantly reduced after administration of chrysin.

Chrysin Administration Attenuated TNBS-Induced Colitis. To confirm the results from the DSS-induced colitis model, we tested the effect of chrysin on some parameters of colitis induced by TNBS, which constitutes a Crohn's disease model. Rectal administration of TNBS dissolved in ethanol induced severe colitis in mice that was characterized by weight loss and diarrhea. Treatment with chrysin once a day, starting 3 days before TNBS administration, significantly reduced body weight loss, ameliorated histologic damage, and improved colon morphology (Fig. 6, A–D).

Discussion

Although the etiology of IBD remains unknown, evidence suggests a complex interplay of intestine commensal bacteria and host immune defense in maintaining healthy homeostasis

Fig. 4. The effects of chrysin on mRNA expressions of NF- κ B target genes in the colon of mice with DSS-induced colitis. mRNA expressions of iNOS (A), ICAM-1 (B), MCP-1 (C), Cox2 (D), TNF- α (E), and IL-6 (F) were determined using qRT-PCR in colon samples isolated from mice (6 per group) treated with vehicle, chrysin, DSS, or chrysin and DSS. Expression was normalized to β -actin, and each bar represents the mean $\pm$ S.D. of triplicates of two independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001, versus DSS-treated group.

of the intestine tract (Medina-Contreras et al., 2011). In particular, recent studies of the xenobiotic sensor nuclear receptor PXR and the inflammatory key mediator NF- κ B have revealed a functional link between xenobiotic neutralization and inflammation and explain how certain xenobiotics can affect the immune response (Zhou et al., 2006).

Activated PXR functions as a xenosensor and regulates the expression of xenobiotic oxidation (e.g., P450s) and conjugation (e.g., UGTs and glutathione S-transferase) enzymes, and transporters (e.g., MDR1, multidrug resistance protein 2, and organic anion transporting polypeptide 2) involved in the metabolism and elimination of potentially harmful chemicals from the body (Chen et al., 2012). PXR is a major regulator of Cyp3a4, a human homolog of rodent Cyp3a11, and is mainly expressed in the adult hepatointestinal system (Gu et al.,

2006). It is estimated that Cyp3a4 is responsible for the metabolism of over 50% of drugs in use today. PXR and its target genes are critical components in intestinal barrier function against xenobiotics and bacteria. For example, PXR and Mdr1 were down-regulated in the colon of patients with IBD (Blokzijl et al., 2007), mice with a genetic deficiency in Mdr1a developed spontaneous colitis (Panwala et al., 1998), and humans with single-nucleotide polymorphisms in PXR gene were associated with a decrease in PXR activity and an increase in susceptibility to IBD (Dring et al., 2006). Our previous study showed that PXR activation was linked to the up-regulation of Cyp3a11 and Mdr1 in the intestine and reduced susceptibility to DSS-induced colitis (Dou et al., 2012). In the current study, chrysin activated both human and mouse PXR in a dose-dependent manner, and consequently, we

Fig. 5. The mechanistic involvement of PXR in the action exerted by chrysin. HepG2 cells were electroporated with PXR siRNA (h) (sc-44057; Santa Cruz Biotechnology) or control siRNA (sc-37007; Santa Cruz Biotechnology). After overnight incubation, cells were treated with chrysin (50 μ M), followed by an additional incubation with or without TNF- α (20 ng/ml). At the end of the incubation, cells were harvested and analyzed by qRT-PCR or Western blot. Data are expressed as mean $\pm$ S.D. of triplicates of two independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001, versus vehicle-treated wells; # P < 0.05; ## P < 0.01; ### P < 0.001, versus TNF- α -treated wells.

observed an up-regulation of PXR target genes, including Cyp3a11 and Mdr1, in the colon mucosa but not in the liver, suggesting its gut-specific activation manner. The minimal effect of chrysin on liver PXR was probably related to its poor absorption (Walle et al., 2001). Because PXR is abundantly expressed in the hepatointestinal system, it is noteworthy that activating PXR in the intestine but not in the liver has clinical benefit for maintaining healthy intestinal barrier and avoids drug-drug interaction caused by altered metabolism profile of drug metabolism enzymes and transporters in the liver (Ma et al., 2007). Further studies should consider the extensive compound metabolism mode and the pharmacokinetics of the

CYP3A substrate to elucidate the intestine-specific PXR activation.

Recently, mutual suppression between PXR and NF- κ B provided a potential molecular mechanism that links xenobiotic metabolism and inflammation (Xie and Tian, 2006; Zhou et al., 2006). Not only does activation of NF- κ B inhibit PXR activity, but activation of PXR inhibits NF- κ B activity and the expression of NF- κ B target genes. For example, pregnenolone-16 α -carbonitrile, a rodent-specific PXR ligand, protected mice from DSS-induced colitis via NF- κ B signaling suppression, indicating the potential value of PXR as a therapeutic target for IBD (Shah et al., 2007). Here, we report that mouse PXR expression inhibited NF- κ B activity and the addition of chrysin enhanced the repression of NF- κ B by PXR-transactivation assay. These data support the fact that ligand activation of PXR inhibits NF- κ B activity (Zhou et al., 2006). The NF- κ B nuclear translocation data performed in mouse macrophage RAW264.7 cells confirmed the role of chrysin in the inhibition of NF- κ B. To further explore the mechanistic involvement of PXR in the action of chrysin, PXR gene expression was silenced by anti-PXR siRNA. We have observed that exposure to TNF- α reduced the expression of PXR-regulated cellular detoxification genes and increased NF- κ B activity in human hepatocyte carcinoma HepG2 cells, whereas these effects were effectively counter-regulated by cotreatment with chrysin. Of interest, the regulatory effect of chrysin was lost in cells lacking the expression of PXR, indicating a key role for PXR in chrysin

TABLE 1

Effects of chrysin on MPO activity and the levels of TNF- α and IL-6 in mice with DSS-induced colitis

Colon segments from mice (6 per group) were excised and homogenized. The supernatants were assayed for the determination of the activity of MPO and the levels of TNF- α and IL-6, as described in *Materials and Methods*. Values are expressed as mean $\pm$ S.D. of triplicates of two independent experiments.

Group	TNF- α	IL-6	MPO
	pg/mg protein	pg/mg protein	U/mg protein
Vehicle	14.6 $\pm$ 1.7	28.9 $\pm$ 2.3	3.7 $\pm$ 0.6
Chrysin	18.3 $\pm$ 1.2	33.2 $\pm$ 2.1	4.4 $\pm$ 0.3
DSS + Vehicle	127.4 $\pm$ 8.5*	214.3 $\pm$ 17.9*	26.8 $\pm$ 2.2*
DSS + Chrysin	47.1 $\pm$ 2.7***	89.4 $\pm$ 6.5**	14.3 $\pm$ 1.9**

* P < 0.001, versus vehicle-treated group; ** P < 0.01, versus DSS-treated group; *** P < 0.001 versus DSS-treated group.

Fig. 6. Protective role of chrysin against TNBS-induced colitis in mice. (A) Body weight changes after TNBS induction of colitis. Data are plotted as percentage of basal body weight. (B) Colon morphology. (C) Representative H&E-stained colon sections (magnification $\times 200$). (D) Histology score. TNBS-induced IBD studies were repeated twice. Values were expressed as mean $\pm$ S.D. of 20 mice in each group. * $P < 0.05$; ** $P < 0.01$, versus TNBS-treated group.

effect. Consistent with the *in vitro* data, *in vivo* evaluation of the protein expressions of phospho-p65, phospho-I κ B α , and I κ B α suggested a direct role of chrysin on NF- κ B inhibition. Actually, preadministration of chrysin not only inhibited NF- κ B activity but also reduced the production of inflammatory cytokines (TNF- α and IL-6), reduced the activity of MPO, down-regulated inflammatory mediators (e.g., iNOS, Cox2, and ICAM-1), and limited the inflammatory (histologic) response. Consequently, these molecular changes resulted in a significant amelioration of DSS- and TNBS-induced colitis. Therefore, in light of available literature on PXR activation and NF- κ B inhibition, our results support the hypothesis that chrysin appears to exert its effect in ameliorating DSS- and TNBS-induced colitis through PXR-mediated NF- κ B inhibition.

Other pathways, distinct from PXR/NF- κ B signaling pathway, are also involved in the pathogenesis of IBD. For instance, baicalein, a natural flavonoid, was shown to abrogate DSS-induced colitis through the caudal-related homeodomain transcription factor 2/PXR pathway in our previous study (Dou et al., 2012). Adachi et al. showed that rosiglitazone, known as antidiabetic drug, decreased the severity of DSS-induced colitis via a peroxisome proliferator-activated receptor- γ activation pathway (Adachi et al., 2006). Shinoda et al. showed that blockade of the Notch pathway contributed to the reduced severity of DSS-induced colitis (Shinoda et al., 2010). Recently, Shin et al. showed that coadministration of chrysin ameliorated the severity of DSS-induced colitis and

antagonized the activity of NF- κ B *in vitro* (Shin et al., 2009). Our study verifies this effect but goes further in providing a deeper molecular basis for such an observation. The present study demonstrated that chrysin behaved as a PXR ligand and decreased the susceptibility of mice to colitis (DSS- and TNBS-induced) via PXR-mediated NF- κ B inhibition mechanism. The data suggest that chrysin unlikely induces systemic drug interactions through PXR activation at a dose used to abrogate intestinal inflammation in mice. In light of the intense attempt in the development of NF- κ B inhibitors and the deleterious adverse effects associated with the ubiquitous expression of NF- κ B, the current study supports the rationale to explore intestine-specific PXR agonist as a therapeutic strategy for IBD because of its hepatointestinal abundant expression profile.

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Authorship Contributions

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