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Chrysin, a natural flavone, improves murine inflammatory bowel diseases

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ABSTRACT

Chrysin (5,7-dihydroxyflavone) is a natural flavone commonly found in many plants. It has previously been shown to be an anti-tumor agent. In this study, we investigated whether chrysin could alleviate the symptoms of dextran sodium sulfate (DSS)-induced colitis in mice and whether chrysin has an inhibitory effect on nuclear factor (NF)-κB activation *in vitro*. A significant blunting of weight loss and clinical signs was observed in DSS-exposed, chrysin-treated mice when compared to vehicle-treated mice. This was associated with a remarkable amelioration of the disruption of the colonic architecture, a significant reduction in colonic myeloperoxidase (MPO) activity, and a decrease in the production of inflammatory mediators such as nitric oxide (NO), prostaglandin (PG) E_2, and pro-inflammatory cytokines. In addition, chrysin inhibited tumor necrosis factor (TNF)-α-induced activation of NF-κB in IEC-6 cells. These findings suggest that chrysin exerts potentially clinically useful anti-inflammatory effects mediated through the suppression of NF-κB activation.

Introduction

Inflammatory bowel disease (IBD) is a group of pathologic conditions of the gastrointestinal tract in humans, of which Crohn's disease (CD) and ulcerative colitis (UC) are the most prominent [1]. UC and CD are associated with intestinal and extra-intestinal clinical manifestations, including weight loss, diarrhea accompanied by blood and/or mucus, fever, gastric dysmotility, and shortening of the colon [2,3]. The immune pathogenesis of IBD is associated with an increase in inflammatory mediators, including reactive oxygen species (ROS) like nitric oxide (NO) [4,5]; prostaglandins (PG) [6,7] and inflammatory cytokines such as tumor necrosis factor (TNF)-α, and interleukin (IL)-1; and IL-6 [8–10]. Although the etiology of IBD still remains unclear, a common feature of IBD is a complex interplay of cells and inflammatory mediators such as cytokines within the intestine [4–11].

Although treatments for IBD, such as glucocorticosteroids, 5-aminosalicylic acid, and immunosuppressive drugs, have been proposed and implemented clinically [12–14], supplementary therapeutic approaches are needed, as many patients either do not respond to the currently available options or demonstrate significant side effects precluding the continued use of these agents. Alternative medicine is becoming an increasingly attractive approach for the treatment of various inflammatory diseases. Among these alternative approaches is the use of food derivatives, which carry the advantage of being relatively non-toxic. However, limited scientific evidence regarding the effectiveness of these natural derivatives and a lack of understanding concerning their action mechanisms have prevented their incorporation into mainstream medical care.

Flavonoids are natural polyphenolic phytochemicals that are ubiquitous in plants and present in the average human diet. Flavonoids are comprised of several classes, including flavonols, flavanones, flavanols, and flavans. Chrysin (5,7-dihydroxyflavone) is a natural flavonoid found in many plant extracts, honey, and propolis [15,16]. Several recent studies have shown that chrysin has multiple biological activities, such as anti-inflammation, anti-cancer, and anti-oxidation effects [17–20]. Chrysin also has the potential for clinical and therapeutic applications against the physiological and biochemical effects of aging [21]. Nevertheless, little is known about the effect of chrysin on inflammatory diseases such as IBD. In this study, we found that chrysin can attenuate IBD by reducing the production of inflammatory mediators.

Materials and methods

Reagents and cell line. All reagents were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise indicated. Dextran sodium sulfate (DSS) (MW 40,000–50,000) was purchased from ICN Biochemicals Inc. (Aurora, OH). RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Hyclone (Logan, UT). Griess reagent was obtained from Promega (Madison, WI). PGE_2 and IL-8 enzyme-linked immunosorbent assay (ELISA) kits were manufactured by R&D Systems (Minneapolis, MN) and

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American Research Products (Belmont, MA), respectively. Multiplex bead array instruments and cytokine kits were purchased from Bio-Rad (Hercules, CA). The antibodies (Abs) used in this study were: anti-inhibitor of NF-κB (Iκ-B) rabbit polyclonal, anti-NF-κB p65 rabbit polyclonal, anti-laminB rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin rabbit polyclonal (Cell Signaling Technology, Danvers, MA). IEC-6 cells from the rat intestinal epithelial cell line were acquired from the Korea Cell Bank (Seoul, Korea).

Mice and experimental protocol. The study protocol was approved by the Animal Care and Use Committee of Hallym University. Six-week-old BALB/c mice were purchased from SLC (Hamamatsu, Japan) and maintained under specific pathogen-free conditions at the animal facility of Hallym University (Chuncheon, Korea). To induce experimental colitis, we treated the mice for 7 days with 5% DSS dissolved in filter-purified water (Millipore Corp., Bedford, MA). The control mice received filtered water alone. Chrysin was dissolved in dimethyl sulfoxide (DMSO) and was freshly diluted in corn oil. Chrysin (1, 5, or 10 mg/kg of body weight) or the vehicle (PBS with DMSO) was administrated orally for 7 days, beginning in coordination with the start of DSS exposure.

Assessment of DSS-induced colitis. The mice were assessed daily for the development of colitis based on body weight, gross rectal bleeding, stool consistency, and survival. Overall disease severity was assessed using a clinical scoring system with a scale of 0–4 [22].

Measurement of cytokines, NO, and PGE_2. Colon tissue culture was performed as described in our previous study [22]. Concentrations of various cytokines in the cell-free culture supernatants of the colon tissues were measure using a Bio-Rad Multiplex bead array instrument and a cytokine kit, according to the manufacturer's protocol. Nitrite and PGE_2 levels in the colon culture medium were measured as described in our previous report [22].

Determination of myeloperoxidase (MPO) activity in the colon. The mouse colons were rinsed with cold PBS, blotted dry, and immediately frozen in liquid nitrogen. They were then stored at −80 °C until they were assayed for MPO activity using the *o*-dianisidine method [23].

Histological analysis. The colonic tissues were fixed in 4% buffered paraformaldehyde, dehydrated through graded concentrations of ethanol, embedded in paraffin, and sectioned. The sections (5 μm thick) were mounted on slides, cleared, and hydrated; and stained with hematoxylin/eosin according to standard protocol.

Immunofluorescence stain. IEC-6 cells were plated onto a chamber slide and incubated under normal culture conditions. When cells reached 70% confluence, chrysin (0–10 μM) was added and incubated for 1 h. Cells were stimulated with recombinant rat TNF-α (R&D Systems, 10 ng/ml) for 30 min and fixed with an ice-cold methanol/acetone (1:1) mixture at −20 °C for 10 min. After washing in PBS, cells were permeabilized with 0.1% Triton X-100 in PBS at room temperature for 20 min. After incubation in blocking buffer (1% bovine serum albumin in PBS) for 10 min, cells were incubated with primary antibodies at 4 °C overnight. After washing in PBS, samples were further incubated with Alexa 488-conjugated secondary antibodies at room temperature for 45 min. In order to stain the nuclei, we added 100 μl of a 1 μg/ml solution of Hoechst 33285 (Molecular Probes, Inc., Eugene, OR) in phosphate-buffered saline at room temperature. Fluorescence photographs were obtained using an AxioImager M1 fluorescence microscope (Carl Zeiss, Gottingen, Germany) with Axiovision software.

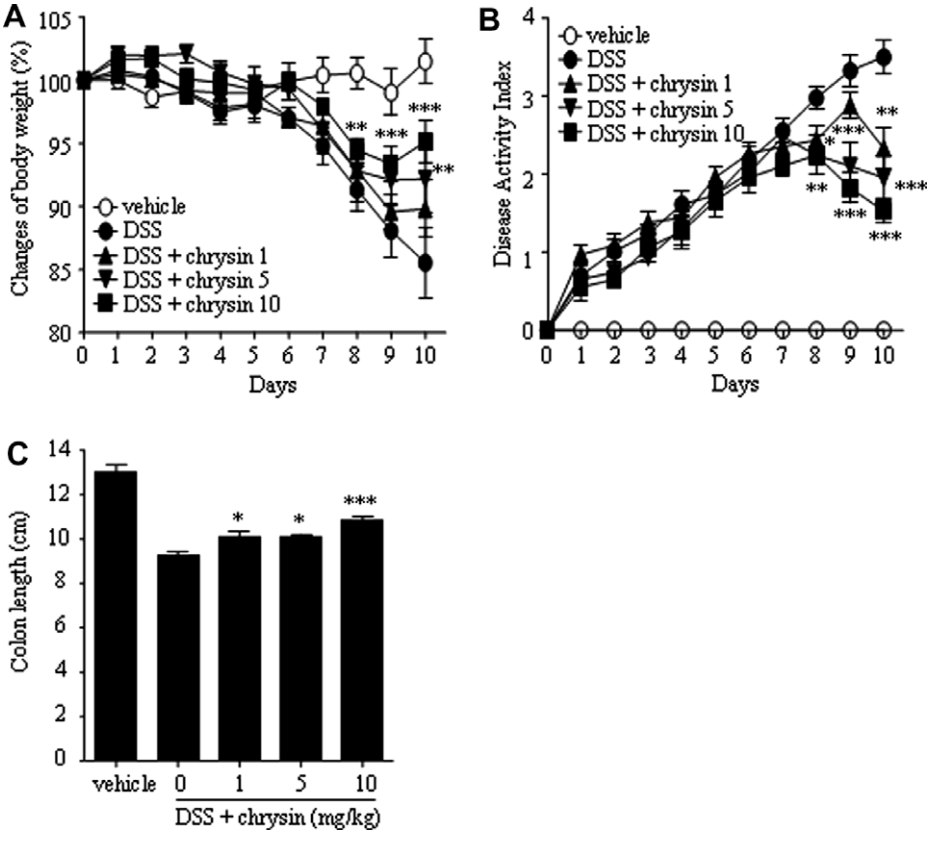

Fig. 1. Chrysin attenuated clinical symptoms in dextran sodium sulfate (DSS)-induced colitis mice. (A) Changes in the body weights and (B) disease activity indices of chrysin-treated mice and vehicle-treated control mice administered 5% DSS were monitored for 10 days. Body weight values are expressed as a percentage of body weight on day 0. (C) Colons were obtained 10 days after DSS administration, and colon lengths were measured. Data are expressed as means ± SEM ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. DSS-treated group; $n = 10$).

Western blot analysis. In order to observe Iκ-Bα degradation and nuclear translocation of NF-κB-p65, we obtained whole cell lysates and cytoplasmic and nuclear extracts and electrophoresed them on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels for the detection of Iκ-Bα and NF-κB-p65.

Statistical analysis. Figures were evaluated using one-way ANOVA, followed by Duncan's multiple range tests. GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA) was used for all calculations. A $P < 0.05$ was considered statistically significant.

Results

Chrysin attenuated body weight loss, clinical symptoms, and shortening of the colon in a DSS-induced murine IBD model

We began monitoring symptomatic colitis parameters such as weight loss and disease activity index (DAI) 10 days after starting oral 5% DSS administration. Mice given 5% DSS in their drinking water for 7 days developed symptoms of colitis without mortality. Compared with vehicle-treated controls, the DSS-administered groups had loose stool or diarrhea, occult or gross rectal bleeding, significantly decreased weight (date not shown and Fig. 1A), markedly increased DAI scoring, and shortening of the colon (Fig. 1B and C). Oral chrysin treatment obviously improved the weight loss and shortening of the colon and reduced DAI scoring.

We undertook histological examination of the colonic sections in order to assess intestinal inflammatory status. When compared to the colon mucosa of vehicle-administered controls, the colonic sections of DSS-treated mice had cryptic distortion, massive inflammatory infiltration and loss of mucous membrane cells, leukocyte infiltration, and lack of integration in the glandular areas (Fig. 2). Conversely, chrysin remarkably attenuated the extent and severity of the histologic signs of cell damage and prevented the various DSS-induced pathologic changes in colitis.

Chrysin inhibited inflammatory mediator production and MPO activity in colonic tissues

NO and PGE_2 are considered important inflammatory mediators that play a key role in the pathogenesis of IBD [5–7]. For this reason, we also evaluated the effect of chrysin on colonic NO and PGE_2 production in DSS-induced colitis mice. As shown in Table 1, DSS administration produced increased levels of NO and PGE_2. However, oral administration of chrysin reduced NO and PGE_2 production in a concentration-dependent manner at day 10.

Table 1
Effects of chrysin on the production of NO, PGE_2 and MPO activity in DSS-induced colitis mice.

	NO (μmol/mg protein)	PGE_2 (ng/mg protein)	MPO activity (U/g tissues)
Vehicle	2.6 ± 0.7	4.3 ± 0.6	4.6 ± 0.3
DSS	18.2 ± 4.6$^{###}$	58.9 ± 12.5$^{###}$	11.3 ± 2.1$^{###}$
DSS + chrysin-1	12.9 ± 2.2*	35.8 ± 5.4**	8.5 ± 0.6***
DSS + chrysin-5	6.2 ± 1.4***	24.2 ± 3.0***	4.1 ± 0.7***
DSS + chrysin-10	6.1 ± 1.5***	21.5 ± 2.3***	3.9 ± 0.4***

Segments of colon from DSS-administered, chrysin-treated mice and from DSS-treated control mice were prepared on day 10 after DSS exposure and cultured without any stimulation for 24 h at 37 °C. Values are means ± SEM ($n = 7$). Statistical significance is based on the difference when compared with DSS-administered mice ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).
$^{###}$ Represents a significant difference between vehicle- and DSS-administered group alone, $P < 0.001$.

MPO is an enzyme produced mainly by polymorphonuclear leucocytes, and it is associated with the degree of neutrophil infiltration in a given tissue [23]. Following 10 days of DSS treatment, MPO activity became markedly increased, to a level approximately 2.5 times higher than that in the control group (Table 1). This increase in MPO activity was significantly reduced by chrysin administration, a finding consistent with the histologic findings. Since MPO activity is considered a biochemical maker of neutrophil infiltration [23], this finding suggests that chrysin exerts anti-inflammatory effects by reducing neutrophil infiltration in the colonic mucosa.

To determine the effect of chrysin on major inflammatory cytokine and chemokine production in the colon, we determined the IL-1β, IL-6, TNF-α, IL-8, monocyte chemoattractant protein (MCP)-1, and keratinocyte chemoattractant (KC) levels (Table 2). After 10 days of DSS administration, the levels of IL-1β, IL-6, and TNF-α had increased significantly. Chrysin administration (5 or 10 mg/kg) prevented significant elevations in the IL-1β and IL-6 levels at day 10. However, the TNF-α level was not reduced by chrysin treatment. Although less than 1 mg/kg of chrysin treatment did not inhibit IL-8, MCP-1, or KC production, 5 or 10 mg/kg of chrysin dramatically reduced IL-8, MCP-1, and KC production

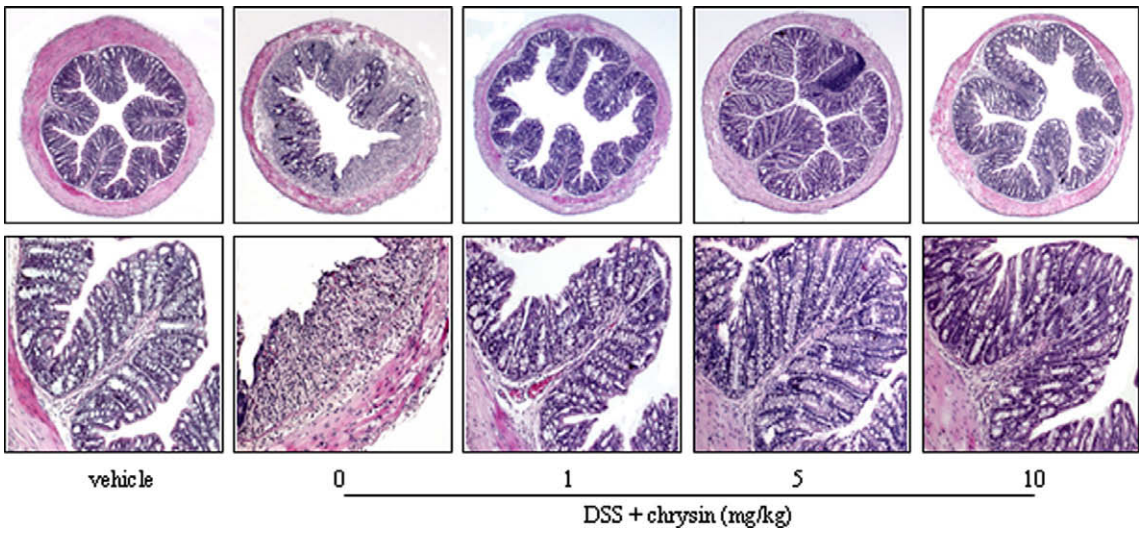

Fig. 2. Histopathologic features of the colon. Colons were obtained 10 days after DSS administration, then sectioned, stained with hematoxylin and eosin, and analyzed histopathologically. Original magnification 40× (upper panel) and 200× (lower panel).

Table 2
Effects of chrysin on the production of colonic inflammatory cytokines and chemokines in DSS-induced colitis mice.

	IL-1β (pg/mg protein)	IL-6 (pg/mg protein)	TNF-α (pg/mg protein)	IL-8 (pg/mg protein)	MCP-1 (pg/mg protein)	KC-1 (pg/mg protein)
Vehicle	2.4 ± 0.4	6.1 ± 2.8	50.8 ± 11.2	18.3 ± 2.9	18.0 ± 8.2	15.6 ± 8.8
DSS	74.0 ± 26.7$^{###}$	170.5 ± 32.9$^{###}$	261.6 ± 47.1$^{###}$	836.6 ± 231.1$^{###}$	315.6 ± 90.6$^{###}$	251.7 ± 49.2$^{###}$
DSS + chrysin-1	31.5 ± 3.7*	86.6 ± 21.5	196.7 ± 53.7	575.4 ± 135.8	215.3 ± 92.8	182.5 ± 52.6
DSS + chrysin-5	23.4 ± 5.6*	66.0 ± 9.4**	191.0 ± 36.8	186.7 ± 66.2**	175.5 ± 76.2	141.3 ± 14.9*
DSS + chrysin-10	21.9 ± 3.7**	40.2 ± 14.5***	213.6 ± 61.6	122.4 ± 50.8***	128.1 ± 50.8*	104.0 ± 5.3***

Segments of colon from DSS-administered, chrysin-treated mice and from DSS-treated control mice were prepared on day 10 after DSS exposure and cultured without any stimulation for 24 h at 37 °C. Values are means ± SEM ($n = 7$). Statistical significance is based on the difference when compared with DSS-administered mice ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).
$^{###}$ Represents a significant difference between vehicle- and DSS-administered group alone, $P < 0.001$.

in the colon (Table 2). This indicates that chrysin exerts anti-inflammatory effects by reducing monocyte and neutrophil infiltration in the colonic mucosa.

Chrysin blocks the degradation of Iκ-B and nuclear translocation of NF-κB in intestinal epithelial cells

Although the precise mechanism of DSS-induced colitis is presently unknown, there are several reports suggesting that intestinal epithelial cells play a key role in regulating the inflammatory reaction via production of various cytokines and chemokines [24–27]. In order to investigate the cellular mechanisms by which chrysin treatment attenuates DSS-induced intestinal inflammation, we evaluated the influences of chrysin on the activation of NF-κB *in vitro* using intestinal epithelial cells (IEC-6 cells). When we investigated the degradation of Iκ-Bα in chrysin (0–10 μM)-treated IEC-6 cells, we observed dramatic, dose-dependent inhibition of Iκ-Bα degradation induced by TNF-α (Fig. 3A). In addition, con-

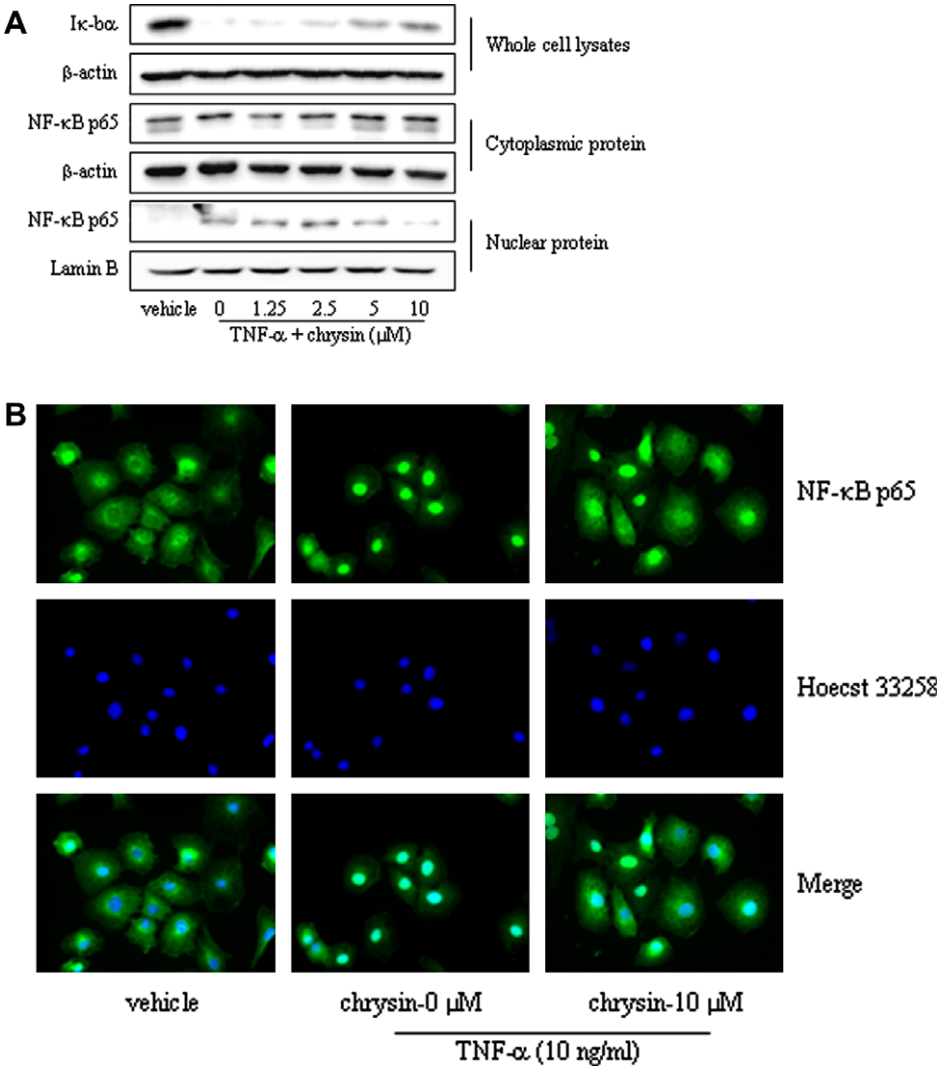

Fig. 3. Chrysin inhibited the degradation of Iκ-Bα and nuclear translocation of NF-κB. IEC-6 cells were preincubated for one hour with increasing chrysin concentrations (0, 1.25, 2.5, 5, 10 μM) and stimulated with 10 ng/ml recombinant rat TNF-α for 30 minutes. (A) Whole cell extracts and cytoplasmic and nuclear extracts were isolated in order to determine the degradation of Iκ-Bα and the expression of NF-κB p65 by western blot analysis, as described in the Materials and methods section. (B) Cells were treated as described in (A), and NF-κB p65 localization was visualised using an anti-NF-κB p65 antibody followed by an Alexa 488-conjugated detection antibody.

sistent with Iκ-Bα degradation, the nuclear translocation of NF-κB p65 was blocked by chrysin treatment in IEC-6 cells (Fig. 3B and C). These results suggest that chrysin at least partially inhibited the TNF-α-induced activation of NF-κB and thereby suppressed inflammatory responses.

Discussion

In this study, we showed that chrysin inhibits Iκ-Bα degradation and nuclear translocation of NF-κB in TNF-α-stimulated intestinal epithelial cells. Furthermore, chrysin alleviates the symptoms of DSS-induced colitis and improves colitis DAI scores by reducing the production of various inflammatory mediators, such as NO, PGE_2, inflammatory cytokines, and chemokines. These results suggest that chrysin has potentially clinically useful anti-inflammatory effects mediated through suppression of NF-κB activation.

NF-κB is the key transcription factor for inflammatory responses and is thought to be important in activation and progression in both humans with IBD and in animals with colitis [28,29]. Indeed, disease activity in mice with colitis is inhibited by antisense oligonucleotides that inhibit the p65 subunit of NF-κB, which suggests a critical role for NF-κB in mediating the inflammatory response [30]. Attempts to control mucosal inflammation through the use of agents that block the NF-κB pathway have met with some success in murine models. For example, it has been shown that NF-κB decoys ameliorate disease severity in several murine experimental colitis models [31]. Chrysin appears to inhibit inflammatory cytokine and chemokine production through downregulation of NF-κB-mediated activation, since NF-κB p65 in the nuclear fraction of TNF-α-treated IEC-6 cells was significantly decreased when they were pretreated with chrysin. NF-κB activation is believed to play a major role in the regulation of pro-inflammatory gene transcription [32], and its suppression by chrysin may inhibit early steps in inflammation and modulate upregulation of multiple pro-inflammatory genes. Binding sites for the NF-κB family of transcription factors are found in the promoter and enhancer regions of multiple genes, including those corresponding to cytokines, chemokines, and growth factors known to be involved in the inflammatory response.

IBD are fairly common chronic inflammatory conditions of the gastrointestinal tract. The main pathologic feature of IBD is an infiltration of polymorphonuclear neutrophils and mononuclear cells into the intestinal tissues. Neutrophil and monocyte migration is in turn triggered by chemotactic bacterial cell-wall products and locally produced cytokines [33]. Measurement of MPO activity has been used as an indicator of neutrophil influx into inflamed gastrointestinal tissue. Our present study showed that MPO activity was remarkably increased in the intestinal tissue of DSS-treated mice compared to that of vehicle-treated control mice, and significantly decreased in mice treated with chrysin; this was consistent with the histologic findings. Inflammatory chemokines direct immune cells to local sites of inflammation [34]. Each chemokine attracts specific cells. For instance, MCP-1 contributes to monocyte recruitment, while KC and IL-8 are specific to neutrophil recruitment, and each plays a crucial role in inflammation [34]. The therapeutic effect exhibited by chrysin may thus be explained by its ability to blunt MPO activity and the production of inflammatory chemokines in DSS-induced murine IBD.

Several previous studies have demonstrated that chrysin regulates the key molecules involved in inflammation, cancer, and aging [17–21,35–37]. For example, chrysin significantly inhibited TNF-α-induced adhesion molecule expression, which is relevant to inflammation and atherosclerosis in human aortic endothelial cells [35]. Lin et al. showed that chrysin inhibits lipopolysaccharide (LPS)-induced angiogenesis via downregulation of vascular endothelial growth factor and IL-6 signaling pathways in human umbilical endothelial cells [36]. In addition, pretreatment of RAW 264.7 macrophage-like cells with chrysin inhibited LPS-induced PGE_2 production without alteration in COX-2 expression [37]. Interestingly, Chen et al. showed that chrysin inhibited intercellular adhesion molecule-1 expression by inhibiting Iκ-B kinase activity, Iκ-B degradation, and NF-κB DNA-protein binding in alveolar epithelial cells (A549 cells) [38].

In conclusion, we demonstrated that chrysin has significant anti-inflammatory effects, alleviating the symptoms and disease scores in DSS-induced colitis and blocking NF-κB activation in TNF-α-stimulated intestinal epithelial cells. Since chrysin is a natural compound with little toxicity, it may have therapeutic potential as a novel anti-inflammatory agent for the management of intestinal inflammation.

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