BIOCHEMICAL PHARMACOLOGY 74 (2007) 870–880

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

2′,4′,6′-Tris(methoxymethoxy) chalcone protects against trinitrobenzene sulfonic acid-induced colitis and blocks tumor necrosis factor-α-induced intestinal epithelial inflammation via heme oxygenase 1-dependent and independent pathways

Sung Hee Lee [a,1], *Dong Hwan Sohn* [a,1], *Xing Yu Jin* [a], *Sang Wook Kim* [b], *Suck Chei Choi* [c], *Geom Seog Seo* [c,*]

[a] *College of Pharmacy, Wonkwang University, Iksan, Jeonbuk 570-749, Republic of Korea*
[b] *Department of Internal Medicine, Chonbuk National University College of Medicine, Jeonju, Jeonbuk 561-712, Republic of Korea*
[c] *Digestive Disease Research Institute, Wonkwang University School of Medicine, Iksan, Jeonbuk 570-749, Republic of Korea*

ARTICLE INFO

Article history:
Received 2 May 2007
Accepted 21 June 2007

Keywords:
2′,4′,6′-Tris(methoxymethoxy) chalcone
Trinitrobenzene sulfonic acid
Tumor necrosis factor-α
Heme oxygenase 1
Nuclear factor-κB

ABSTRACT

2′,4′,6′-Tris(methoxymethoxy) chalcone (TMMC), a synthesized chalcone derivative, displays potent antiproliferative and anti-inflammatory effects in rat hepatic stellate cells and murine macrophages, respectively. Here we tested the hypothesis that TMMC could ameliorate diseases characterized by mucosal inflammation. Treatment of mice with TMMC significantly protected against trinitrobenzene sulfonic acid (TNBS)-induced colitis, as assessed by reductions in the weight loss, colonic damage and mucosal ulceration that together characterize this symptom. Moreover, TMMC suppressed the expression of intercellular adhesion molecule-1, interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α) in the mice treated with TNBS. Pretreatment of human intestinal epithelial HT-29 cells with TMMC also significantly inhibited the IL-8 and extracellular matrix metalloproteinase-7 levels induced by TNF-α. TMMC induced the expression of heme oxygenase 1 (HO-1) in HT-29 cells. TMMC increased extracellular signal-regulated kinase1/2 and p38 kinase phosphorylation levels, which led to the nuclear translocation of nuclear factor-erythroid 2-related factor 2 (Nrf2) and consequently to HO-1 expression. TMMC inhibited TNF-α-induced nuclear factor κB (NF-κB) activation directly and indirectly. Interestingly, the latter is mediated by HO-1, which presumably blocks the TNF-α-induced nuclear translocation of NF-κB p65 without affecting I-κBα degradation. Moreover, we found that the different products of HO-1, carbon monoxide and bilirubin, exerted anti-inflammatory effects that were additive or synergistic in HT-29 cells stimulated with TNF-α. Thus, TMMC might serve to protect against intestinal inflammatory diseases.

* *Corresponding author*. Tel.: +82 63 859 2565; fax: +82 63 855 2025.
E-mail address: medsgs@wonkwang.ac.kr (G.S. Seo).

[1] These authors contributed equally to this work.

Abbreviations: CO, carbon monoxide; Erk1/2, extracellular signal-regulated kinase1/2; HO-1, heme oxygenase; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; MAPK, mitogen-activated protein kinase; MMP, matrix matalloproteinase; NF-κB, nuclear factor κB; Nrf2, nuclear factor-erythroid 2-related factor 2; SnPP, Tin protoporphyrin; TMMC, 2′,4′,6′-tris(methoxymethoxy) chalcone; TNBS, trinitrobenzene sulfonic acid

0006-2952/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2007.06.034

1. Introduction

Inflammatory bowel disease (IBD) is a chronic condition of the intestine of unknown etiology involving multiple immune, genetic and environmental factors, with only suboptimal medical and surgical therapies available [1]. Studies elucidating the pathophysiological mechanisms involved in these disorders suggest that they entail general inflammatory processes, resulting in tissue damage and clinical manifestations [2]. In an experimental model using intracolonic administration of trinitrobenzene sulfonic acid (TNBS), the intestinal inflammation induced resembles many of the clinical, histopathological and immune characteristics of IBD in humans [3,4].

Heme oxygenase (HO) is the rate-limiting enzyme in the catabolism of excess heme and generation of iron, carbon monoxide (CO) and biliverdine, which is subsequently converted to bilirubin by biliverdine reductase [5]. As a redox-sensitive inducible protein, HO-1 has been implicated in serving as a protective gene because of its abilities in antioxidant, anti-inflammatory and anti-apoptotic actions [6–8]. HO-1 is abundant in the epithelial cells of the rat and human intestine [9,10]. In addition, both mice and human deficient in HO-1 gene expression have a phenotype of an increased inflammatory state [11,12]. In view of the growing evidence that HO-1 provides anti-inflammatory protection to the intestine [13–15], HO-1 induction by pharmacological modulators may represent a novel target for therapeutic intervention.

We have shown previously that 2′,4′,6′-tris(methoxymethoxy) chalcone (TMMC), a synthetic chalcone derivative, displays potent anti-inflammatory and antiproliferative effects; these are mediated by the induction of HO-1 expression in murine macrophages and rat hepatic stellate cells, respectively [16,17]. In the present study, we employed a murine TNBS-induced colitis model to determine whether TMMC is protective against this disorder. In addition, we tested the potency of TMMC as an intestinal epithelial inducer of HO-1 expression, and its regulation in HT-29 cells.

2. Materials and methods

2.1. Reagents and cell culture

All reagents were from Sigma–Aldrich (St. Louis, MO) unless otherwise indicated. Tumor necrosis factor-α (TNF-α) was from R&D Systems (Minneapolis, MN). Inhibitors of p38 kinase (SB203580), of extracellular signal-regulated kinase1/2 (Erk1/2) kinase (PD98059) and of SAPK/c-Jun N-terminal kinase (JNK) kinase (SP600125) were from Calbiochem (San Diego, CA). Tin protoporphyrin (SnPP), an inhibitor of heme oxygenase activity, was from Porphyrin Products Inc. (Logan, UT, USA). The HT-29 cell lines used in this work were obtained from the American Type Culture Collection (Rockville, MD) and cultured at 37 °C under 5% CO_2 in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with antibiotics (100 IU/ml of penicillin G and 100 μg/ml of streptomycin) and 10% heat-inactivated fetal bovine serum (Gibco-BRL, Gaithersburg, MD).

2.2. Interleukin-8 (IL-8) analysis

IL-8 protein secretion was measured in cell culture supernatant by enzyme-linked immunosorbent assay (ELISA; R&D Systems) according to the manufacturer's instructions. Measurements were performed in triplicate.

2.3. Experimental colitis

The Animal Studies Ethics Committee of Wonkwang University approved all of the experiments. Male BALB/c mice (7–8-week-old) were purchased from Dae-Han Bio Animal Center Company (Daejun, Republic of Korea). Mice were anesthetized lightly using diethyl ether. Using a polyethylene catheter fitted onto a 1 ml syringe, we slowly injected 0.1 ml of 50% (v/v) ethanol containing 2 mg TNBS (two doses separated by a seven-day interval) into the lumen of the colon (3.5 cm proximal to the anus through the rectum). Animals were kept in a vertical position for 60 s and returned to their cages. Animals in the control group were handled similarly, but 50% (v/v) ethanol alone was administrated instead. All mice were sacrificed on day 9 after the first TNBS administration. To investigate the effect of TMMC, mice were administrated TMMC at doses of 100 or 200 μg/mouse per day via the lumen of the colon one day before, and three and six days after the first TNBS administration.

2.4. Histological and immunohistochemical analysis

For histology, tissues were fixed in 10% formalin and paraffin-embedded tissue sections were stained with H&E using standard techniques. Immunohistochemistry was performed using the LSAB® 2kit (Dako), based on the streptavidin–biotin–peroxidase complex method. Following endogenous peroxidase blocking, the sections were incubated at room temperature for 2 h with anti-intercellular adhesion molecule (ICAM)-1, anti-IL-1β and anti-TNF-α antibodies.

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA (5 μg) was reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Gibco-BRL) and the resultant cDNA was diluted 10-fold for PCR. Oligonucleotide primer sequences were as follows: human IL-8 sense, 5′-ttc ctg att tct gca gct ctg tgt-3′ (corresponding to nt 77–100) and antisense, 5′-ctc agc cct ctt caa aaa ctt ctc-3′ (corresponding to nt 308–331), according to GenBank accession no. **BC013615**. PCR reactions were carried out in the presence of 1.5 mM $MgCl_2$ for 30 cycles at the following temperatures and times: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min; these cycles were followed by a final extension at 72 °C for 10 min. The amplified PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining. The integrity of cDNA samples was confirmed using primers specific for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sense, 5′-cgg agt caa cgg att tgg tcg tat-3′ corresponding to nt 888–911: antisense, 5′-agc ctt ctc cat ggt ggt gaa gac-3′ corresponding to nt 1171–1194, according to GenBank accession no. **BC014085**).

2.6. Western blot analysis

Equal volumes of conditioned medium from HT-29 cells were concentrated 10-fold in 10 kDa microcentrifuge concentrators (Millipore, Bedford, MA). Equal amounts of protein were resolved on 10% SDS-polyacrylamide gel by electrophoresis, blotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) and incubated with anti-matrix metalloproteinase 7 (MMP-7) antibody (Chemicon International).

Whole-cell lysates (for HO-1, mitogen-activated protein kinase [MAPK], ICAM-1 and cyclooxygenase 2 [COX-2]), nuclear extracts (for p65, nuclear factor-erythroid 2-related factor 2 [Nrf2] and TATA binding protein [TBP]), or cytosolic extracts (for phospho-I-κBα and I-κBα) were separated by 10% SDS-polyacrylamide gel electrophoresis. Nitrocellulose membranes were incubated with specific antibodies against HO-1, I-κBα, p65, Nrf2, ICAM-1, COX-2 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies against phospho-Erk1/2, -p38 and -I-κBα were obtained from Cell Signaling Technology (Beverly, MA). TBP (Abcam, UK) was used as a nuclear protein loading control. Immunoreactive bands were detected by incubating with anti-rabbit, anti-goat, or anti-mouse IgG antibodies conjugated with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

2.7. Statistical analysis

Data were analyzed using Student's *t*-test when appropriate or by a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests when comparing more than three means. Calculations were performed with GraphPad Prism software (GraphPad Software, San Diego, CA).

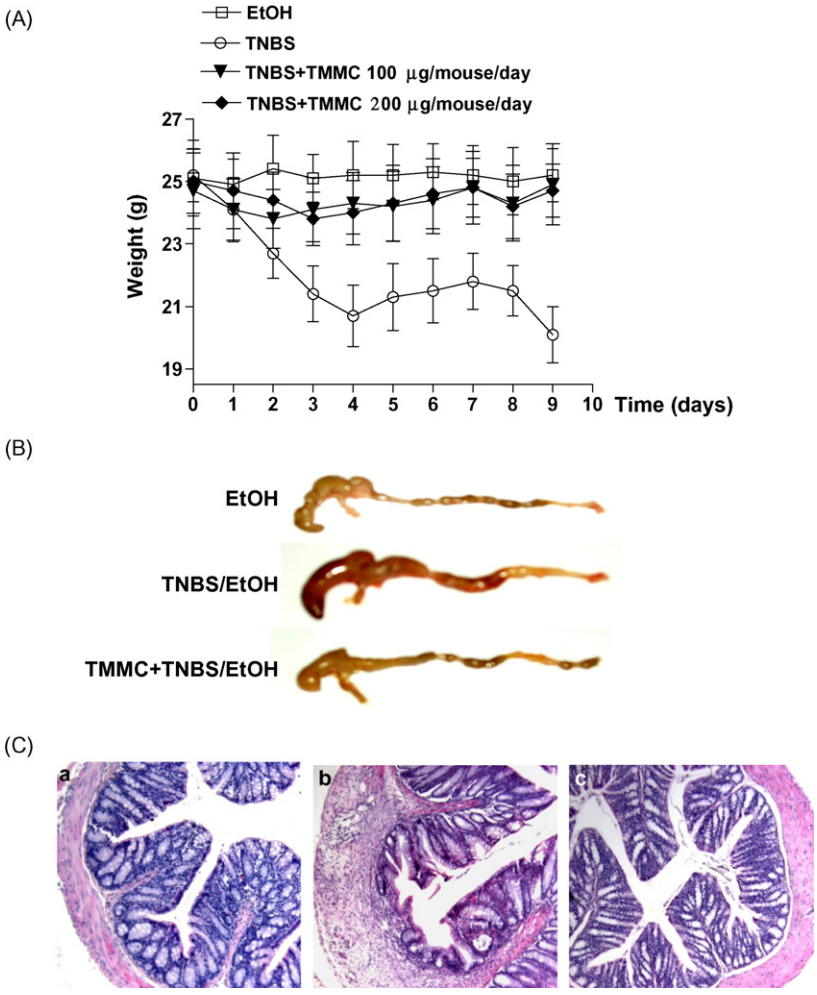

Fig. 1 – TMMC ameliorates the clinical, macroscopic and histopathological features of TNBS-induced colitis. Colitis was induced by rectal administration of two doses of TNBS in 50% ethanol (EtOH), seven days apart. Mice treated with 50% EtOH alone were used as controls. TMMC (100 or 200 μg/mouse per day) was infused into the lumen of the colon one day before and three and six days after the first TNBS treatment. All mice were sacrificed on the ninth day after the first TNBS administration. The clinical evaluation and severity of colitis were monitored by mouse weight changes (A), macroscopic signs of disease (B) and histopathology (C). The weight changes (A) are expressed as the mean ± S.D. of five mice per group. (C) EtOH-treated control mice (a), TNBS/EtOH-treated mice (b), TMMC + TNBS/EtOH-treated mice (c).

3. Results

3.1. *Protective effects of TMMC against TNBS-induced colitis in mice*

Mice treated with TNBS in 50% ethanol developed severe bloody diarrhea and rectal prolapse accompanied by an extensive wasting disease as assessed nine days after the first TNBS challenge. Control mice treated with 50% ethanol alone failed to develop colitis and had a healthy appearance. After administration of TNBS, a dramatic and rapid decrease in body weight was observed from the colitis and was maintained during the nine-day period (Fig. 1A). However, administration of TMMC significantly attenuated body weight loss associated with TNBS-induced colitis ($P < 0.05$). Macroscopic inspection of the colon showed evidence of severe edema and hemorrhage, compared with control colons from ethanol-treated mice (Fig. 1B). Treatment with TMMC almost completely prevented both edema and inflammation in the colon. Histology of the colon from ethanol-treated mice showed normal features (Fig. 1C(a)). We assessed focal ulceration of the colonic mucosa extending through the muscularis mucosae as well as desquamated areas or loss of the epithelium. The architecture of the crypts was distorted and the lamina propria was thickened in peripheral areas of distorted crypts, especially in basal areas (Fig. 1C(b)). However, TMMC treatment caused an attenuation of these signs of cell damage and inflammatory cells were not found in the lamina propria (Fig. 1C(c)). These observations suggest the beginning of re-epithelization and healing in the TMMC-treated mice.

To investigate whether the protective effect of TMMC in TNBS-induced colitis was also associated with the down-regulation of ICAM-1, IL-1β and TNF-α levels, immunohistochemical staining was performed in the local colonic tissue. This revealed increased immunoreactivity for all these three proteins in TNBS-treated mice compared with control mice. Levels of these three proteins were reduced in colonic samples of mice treated with TMMC. As shown in Fig. 2, TMMC administration dramatically reduced ICAM-1, IL-1β and TNF-α levels in the mice with TNBS-induced colitis.

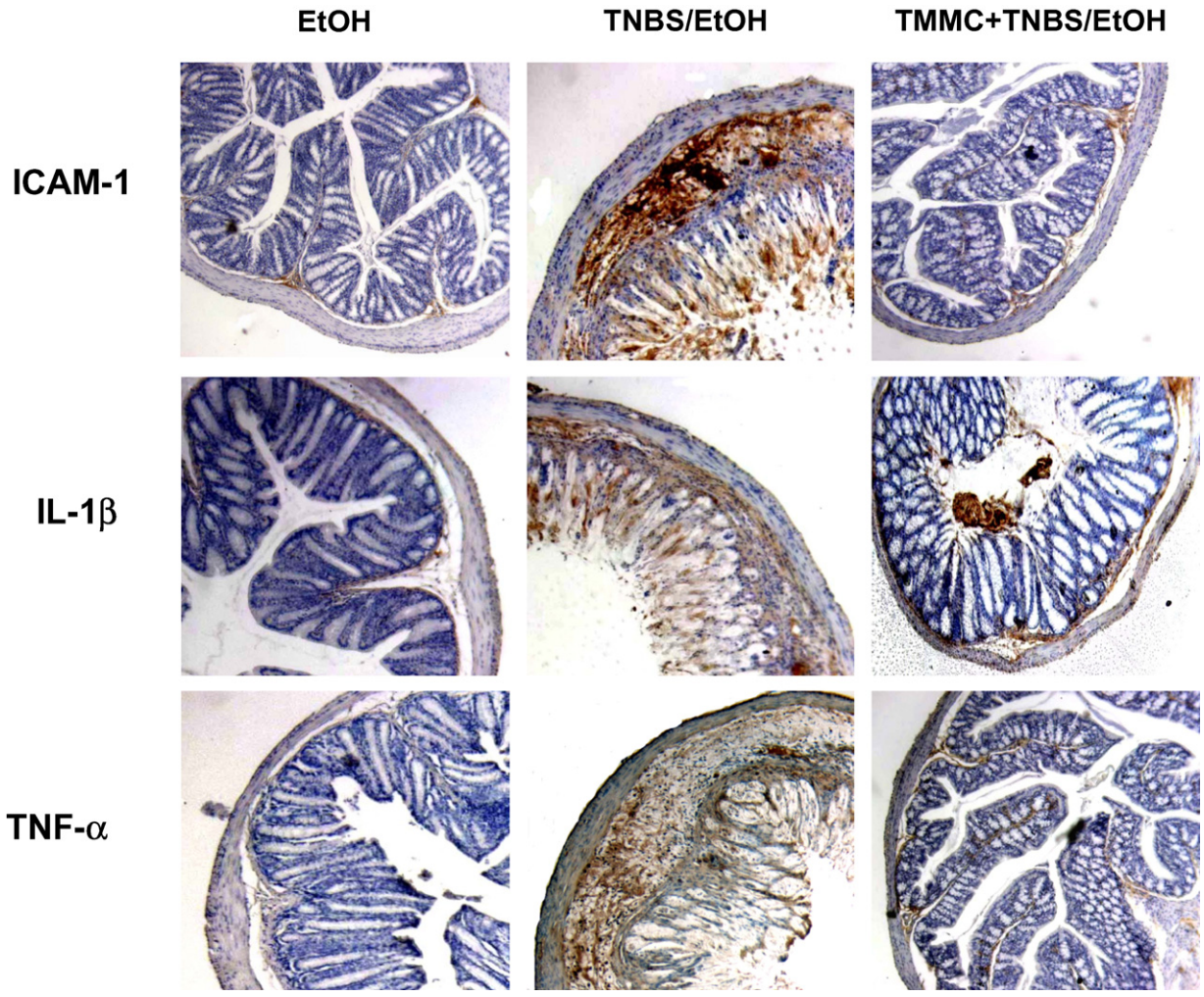

Fig. 2 – TMMC reduces the expression of ICAM-1, IL-1β and TNF-α in colonic mucosa of mice with TNBS-induce colitis. Tissue sections from the experimental groups were incubated with primary antibodies (anti-ICAM-1, -IL-1β, or -TNF-α). The slides were then incubated with anti-rabbit or anti-goat biotinylated secondary antibodies and stained with 3,3′-diaminobenzidine and hematoxylin (original magnification 100×).

3.2. TMMC inhibits TNF-α-induced IL-8 and MMP-7 levels in HT-29 cells

The effects of TMMC on intestinal inflammation and associated pathways were investigated using human origin intestinal epithelial cell (IEC) HT-29 cells. We first examined the effect of TMMC on IEC proinflammatory cytokine levels. The level of IL-8 produced by HT-29 cells was significantly increased by the addition of TNF-α. We therefore examined whether TMMC could suppress TNF-α-induced IL-8 gene expression. HT-29 cells were pretreated with various doses of TMMC and then stimulated with TNF-α for 12 h, after which IL-8 mRNA accumulation was analyzed by RT-PCR. It was indeed induced by TNF-α and was significantly decreased in cells treated with 5 or 10 μM TMMC (Fig. 3A, *upper panel*). Inhibition of IL-8 gene expression was confirmed by measuring IL-8 protein production using ELISA. Addition of TMMC also dramatically reduced TNF-α-induced IL-8 secretion in a dose-dependent manner (Fig. 3B).

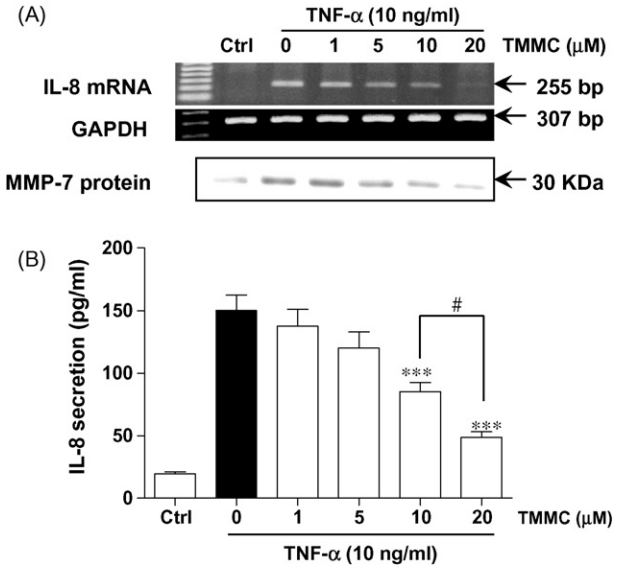

Fig. 3 – (A) Effects of TMMC on TNF-α-induced IL-8 mRNA (*upper panel*) and extracellular MMP-7 protein (*lower panel*) levels. Cells were pretreated with TMMC for 1 h and then the cells were further incubated for 12 h (for IL-8 mRNA) or 24 h (for MMP-7 protein) with TNF-α (10 ng/ml). Total RNA was extracted and the level of IL-8 mRNA was determined by RT–PCR. The amount of RNA loaded in each lane was confirmed using human GAPDH as a reference. The concentrated conditioned media were collected and extracellular MMP-7 protein level determined by immunoblotting. (B) TMMC inhibits the secretion of IL-8 in HT-29 cells. Cells were pretreated for 1 h with increasing concentrations of TMMC before the start of the 24 h incubation with TNF-α (10 ng/ml). IL-8 protein secretion was determined by ELISA. Results are the mean ± SD of three independent experiments, each performed using triplicate wells. $P < 0.001$ vs. cells treated with TNF-α alone, as analyzed by one-way ANOVA and Tukey's multiple comparison tests. $^{\#}P < 0.05$, as analyzed by Student's *t*-test.

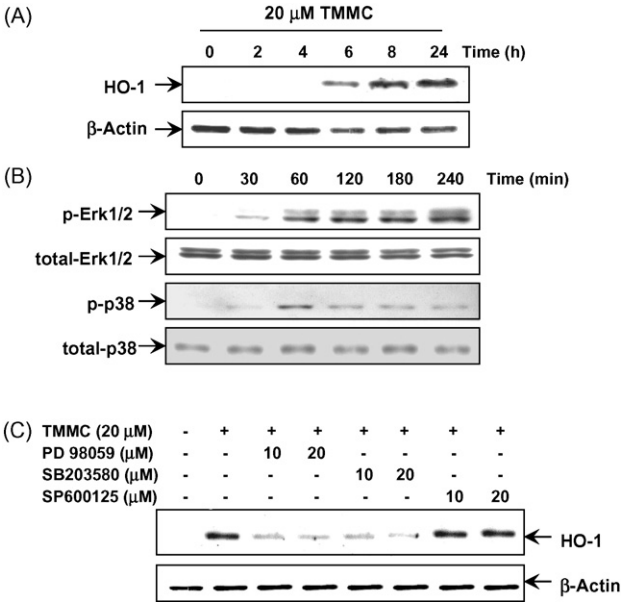

Fig. 4 – (A) Time-dependent induction of HO-1 protein expression by TMMC. HT-29 cells were treated with 20 μM TMMC for 24 h and the levels of HO-1 protein in cells were analyzed by western blotting; β-actin is shown as the loading control. (B) Time-course experiment on the effects of TMMC on Erk1/2 and p38 phosphorylation levels. Cells were treated with TMMC (20 μM) for the indicated periods (30, 60, 120, 180 or 240 min). Cell lysates were obtained and separated by electrophoresis, transferred to nitrocellulose and then incubated with anti-phospho-Erk1/2 or -p38 antibodies. Equal loading of proteins in each lane was confirmed by probing the membrane with anti-total-Erk1/2 or -p38 antibodies. (C) Effect of MAPK inhibition on TMMC-induced HO-1 gene expression in HT-29 cells. Cells were pretreated with the indicated concentrations of PD98059, SB203580 or SP600125 for 1 h and then treated with 20 μM TMMC for 8 h. The levels of HO-1 protein were detected by western blotting.

We examined whether TMMC played any role in the secretion of extracellular MMP-7 in TNF-α-stimulated HT-29 cells. TMMC significantly decreased TNF-α-stimulated MMP-7 production in a dose-dependent manner (Fig. 3A, *lower panel*). A concentration of 20 μM of TMMC inhibited the secretion of IL-8 and MMP-7 maximally ($P < 0.05$ versus 10 μM TMMC), so we used that concentration for the following experiments, unless otherwise indicated.

3.3. TMMC-induced HO-1 protein expression involves phosphorylation of both Erk1/2 and p38 in HT-29 cells

As we have shown that TMMC induces HO-1 expression in murine macrophages and rat hepatic stellate cells [16,17], we tested whether TMMC had such an action in HT-29 cells. As shown in Fig. 4A, at 20 μM, TMMC induced a time-dependent increase in HO-1 protein levels that was apparent 6 h after treatment and peaked at 24 h. TMMC also induced HO-1 protein expression in a concentration-dependent manner

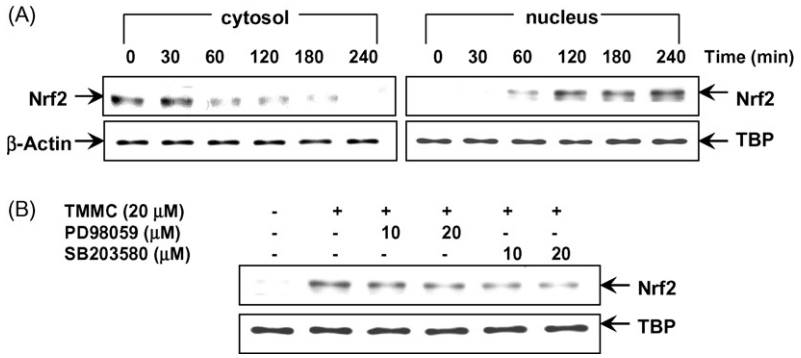

Fig. 5 – Effects of TMMC on Nrf2 nuclear translocation in HT-29 cells. (A) Cells were treated with 20 μM TMMC for the indicated periods (30, 60, 120, 180 or 240 min). Cytoplasmic and nuclear extracts were subjected to western immunoblotting with anti-Nrf2, -β-actin, or -TBP antibodies. Anti-TBP antibody was used as a nuclear protein loading control. (B) Effects of TMMC-induced Erk1/2 and p38 activation on Nrf2 nuclear translocation. Cells were incubated with TMMC for 2 h in the presence or absence of PD98059 or SB203580 and then nuclear extracts were subjected to western blotting analysis using an anti-Nrf2 antibody.

(data not shown). In mammalian cells, Erk1/2, JNK and p38 MAPK represent the three major kinase cascades. All of these appear to be involved to some extent in the upregulation of HO-1 gene expression in response to diverse stimuli [18–20]. Therefore, we examined the effects of TMMC on MAPK activation in HT-29 cells. Treatment with 20 μM TMMC increased the activities of Erk1/2 and p38 MAPK (Fig. 4B), but not JNK (data not shown). Increased phosphorylations of Erk1/2 and p38 were detected within 60 min after treatment with TMMC and the amount of these two kinases remained above the basal level for 4 h. To further confirm the possible upstream signaling pathway involved in the TMMC-mediated HO-1 expression, the cells were pretreated with the Erk1/2 inhibitor PD98059, the p38 inhibitor SB203580, or with the JNK inhibitor SP600125. Inhibitors for MAPK used in this study had no significant cytotoxicity (data not shown). Suppression of Erk1/2 or p38 activities, but not of JNK MAPK activity, abolished the TMMC-induced HO-1 expression (Fig. 4C). This data confirm the involvement of Erk1/2 and p38 signaling in HO-1 gene expression by TMMC in HT-29 cells.

3.4. *TMMC-induced HO-1 protein expression involves nuclear translocation of Nrf2 in HT-29 cells*

It has been reported that Nrf2 is essential in protecting intestinal integrity through the regulation of proinflammatory cytokines [21] and is an important upstream step of HO-1 gene expression [22,23]. Thus, we examined whether TMMC could induce Nrf2 nuclear translocation in HT-29 cells. Nrf2 proteins in the cytoplasm and nuclear compartments of the cells were subjected to gel electrophoresis. Western blot analysis revealed that TMMC significantly increased Nrf2 nuclear translocation in a time-dependent manner, whereas Nrf2 protein levels in the cytoplasm decreased gradually (Fig. 5A). Our findings suggest that the Erk1/2 and p38 pathways are involved in the regulation of TMMC-induced HO-1 gene expression (Fig. 4B and C). Therefore, using specific inhibitors of Erk1/2 and p38 MAPK, we evaluated whether TMMC-induced Erk1/2 and p38 phosphorylations might be involved in TMMC-induced Nrf2 nuclear translocation. Blocking the phosphorylation of Erk or p38 inhibited TMMC-induced Nrf2 translocation in dose-dependent manners (Fig. 5B). These results suggest that activation of Erk1/2 or p38 might be central to the nuclear translocation of Nrf2 by TMMC in HT-29 cells.

3.5. *HO-1 mediates the inhibition of IL-8 production by TMMC*

To clarify the contribution of HO-1 in TMMC-mediated IL-8 downregulation in TNF-α-stimulated HT-29 cells, we tested the action of hemin [24], a potent HO-1 inducer. The cells were treated with TMMC with or without hemin in the presence of TNF-α for 24 h and IL-8 levels were analyzed (Fig. 6A). Induction of HO-1 protein expression by hemin was able to potentiate the inhibitory effects on TNF-α-induced IL-8 production and there was an additive effect of hemin and TMMC on TNF-α-induced IL-8 production. We cotreated HT-29 cells with TMMC, with or without SnPP, as an inhibitor of HO-1 activity [16,17], in the presence of TNF-α and the amount of IL-8 production was measured. This treatment significantly attenuated their inhibitory activity of TMMC on TNF-α-induced IL-8 production (Fig. 6B). Thus, expression of the HO-1 gene might participate in the inhibitory mechanism of TMMC on TNF-α-induced IL-8 production.

3.6. *TMMC inhibits TNF-α-induced NF-κB activation directly and indirectly*

We examined whether TMMC-induced HO-1 expression might be related to NF-κB activity. Because TMMC-induced HO-1 expression was evident even 6 h after treatment with TMMC (Fig. 4A), TMMC was added for different times (1 h or 7 h) before the introduction of TNF-α and then Erk1/2 and p38 phosphorylation levels, and I-κBα degradation and NF-κB p65 nuclear translocation were determined by western blot analysis. TMMC-treated cells induced Erk1/2 and p38 phosphorylation levels beyond those induced by TNF-α alone at both times of pretreatment. We suggest that further increase in Erk1/2 and p38 phosphorylations by TMMC may be associated with HO-1 gene expression.

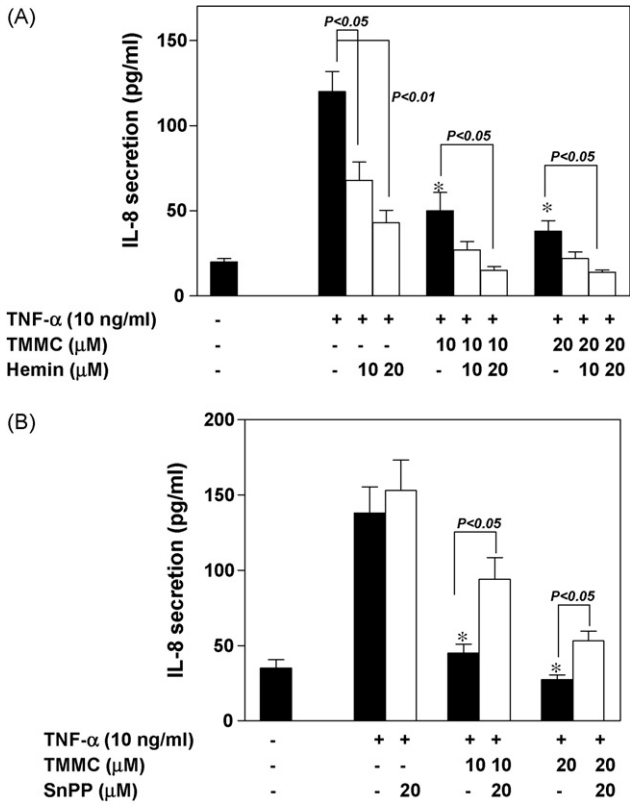

Fig. 6 – (A) Hemin enhances the inhibitory activity of TMMC on TNF-α-induced IL-8 production. Cells were treated with hemin (10 and 20 μM) and TMMC (10 and 20 μM), in the presence of TNF-α (10 ng/ml). IL-8 protein secretion was determined by ELISA. Data were obtained from three independent experiments and are expressed as the mean ± SD. *P < 0.01 vs. TNF-α-only treated cells. (B) Tin protoporphyrin (SnPP) attenuates the inhibitory effects of TMMC on TNF-α-induced IL-8 production in HT-29 cells. Cells were treated with TMMC (10 and 20 μM) and TNF-α (10 ng/ml) in the presence or absence of SnPP (20 μM) and the amount of IL-8 was measured. Data were obtained from three independent experiments and are expressed as the mean ± S.D. *P < 0.01 vs. cells treated with TNF-α alone.

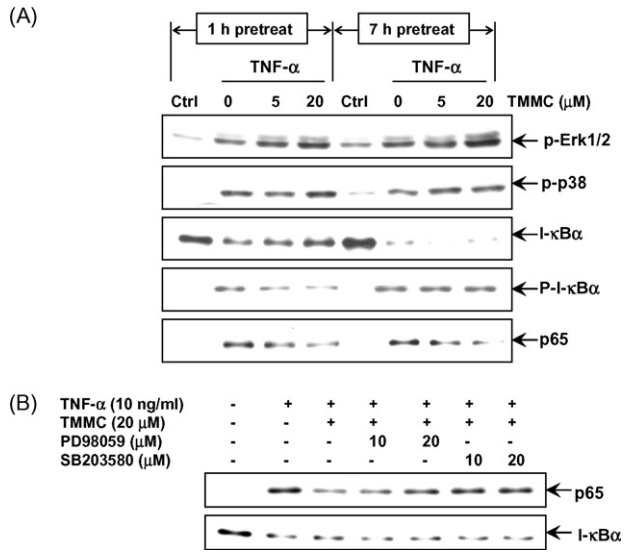

Fig. 7 – (A) Preincubation with TMMC for different durations alters the levels of I-κBα phosphorylation and degradation in TNF-α-stimulated HT-29 cells. Cells were incubated with 20 μM TMMC for different times (1 or 7 h) and then stimulated with 10 ng/ml TNF-α. After 15 min, cell lysates were extracted and then analyzed by western blotting with anti-phospho-Erk1/2 or -p38 antibodies. In addition, cytoplasmic and nuclear extracts were subjected to western immunoblotting with anti-phospho-I-κBα, -I-κBα or -p65 antibodies. (B) TMMC-induced HO-1 protein inhibits TNF-α-induced nuclear translocation of NF-κB p65 without affecting I-κBα degradation. To block TMMC-induced HO-1 expression, cells were pretreated with PD98059 or SB203580 for 1 h, incubated for 7 h with TMMC and then stimulated with 10 ng/ml TNF-α. After 15 min, cytoplasmic and nuclear extracts were subjected to western immunoblotting with anti-I-κBα or -p65 antibodies, respectively.

Although HO-1 protein expression was not observed (Fig. 4A), pretreatment with TMMC for 1 h reduced I-κBα degradation and inhibited nuclear localization of the p65 protein by TNF-α stimulation in HT-29 cells. Thus, TMMC inhibits TNF-α-induced inflammation via a direct inhibition of NF-κB activation without being associated with HO-1 gene expression. Moreover, HT-29 cells were pretreated for 7 h with the indicated concentrations of TMMC and then exposed to TNF-α. TMMC inhibited the TNF-α-induced nuclear translocation of NF-κB p65 in a dose-dependent manner. However, under the same conditions, TMMC did not inhibit TNF-α-induced I-κBα degradation (Fig. 7A). To confirm this result, phosphorylation levels of I-κBα at Ser-32 and Ser-36 residues were also analyzed by western blotting. Pretreatment with TMMC for 7 h did not inhibit TNF-α-induced I-κBα phosphorylation. As shown in Fig. 4, the Erk1/2 and p38 pathways are involved in the regulation of HO-1 gene expression by TMMC. Therefore, to test whether TMMC-induced HO-1 inhibits TNF-α-induced nuclear translocation of NF-κB p65, cells were treated with TMMC in the presence of PD98059 or SB203580 for 7 h and then stimulated with TNF-α. Western blotting analyses for NF-κB p65 and I-κBα were carried out using nuclear and cytoplasmic extracts. Blocking Erk1/2 or p38 phosphorylation inhibited TMMC-induced HO-1 expression (data not shown) and reversed the inhibition of TMMC on TNF-α-induced NF-κB p65 translocation, while these inhibitors did not affect I-κBα degradation. These data suggest that TMMC-induced HO-1 protein interferes with TNF-α-induced NF-κB activation, especially the nuclear translocation step of NF-κB p65.

3.7. *Bilirubin and CO downregulate the inflammatory response*

As HO-1 participated in the inhibitory mechanism of TNF-α-induced IL-8 production (Fig. 6) and NF-κB activation (Fig. 7), we next investigated whether its metabolites, CO and bilirubin, could also inhibit inflammation, in terms of the inhibition of

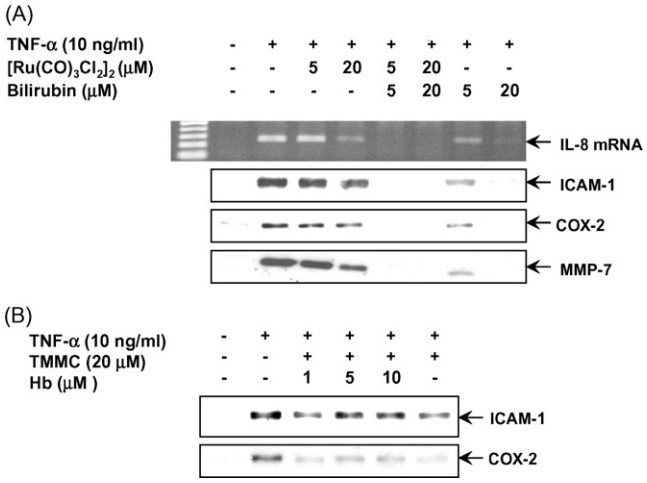

Fig. 8 – (A) Effects of the tricarbonyldichlororuthenium (II) dimer ($[Ru(CO)_3Cl_2]_2$) and bilirubin on IL-8, ICAM-1, COX-2 and extracellular MMP-7 levels in TNF-α-stimulated HT-29 cells. Cells were preincubated for 1 h with $[Ru(CO)_3Cl_2]_2$ and/or bilirubin and stimulated with TNF-α (10 ng/ml). IL-8 mRNA expression was measured by RT–PCR. ICAM-1, COX-2 and MMP-7 gene expression levels were measured by western immunoblotting. (B) CO mediates the suppression of TNF-α-induced ICAM-1 and COX-2 expression in HT-29 cells treated with TMMC. Cells were incubated without or with 20 μM TMMC for 1 h, followed by treatment without or with hemoglobin (Hb) for an additional 1 h before TNF-α (10 ng/ml) stimulation for 24 h.

IL-8, ICAM-1, COX-2 and MMP-7 expression. The CO donor tricarbonyldichlororuthenium (II) dimer (RuCO, up to 20 μM) and bilirubin (up to 20 μM) used in this study had no significant cytotoxic activity (data not shown). As shown in Fig. 8A, RuCO and bilirubin suppressed the TNF-α-induced expression levels of the above four genes dose-dependently. This suggests that CO and bilirubin generated by HO-1 may be the main anti-inflammatory molecules in HT-29 cells. The combination of both treatments reduced the four gene expression levels nearly to control (Fig. 8A, lanes 5 and 6). Next, to confirm the synergetic effect of CO and bilirubin, we examined the effects of TMMC, an HO-1 inducer, on ICAM-1 and COX-2 levels in the presence of hemoglobin – a scavenger for CO – in TNF-α-stimulated HT-29 cells. Hemoglobin effectively reversed the inhibition of TNF-α-induced ICAM-1 and COX-2 gene expression levels by treatment with 20 μM TMMC in dose-dependent manners. However, in the presence of this CO scavenger, inhibition of TMMC on these two expressions was also observed, compared with cells treated with TNF-α alone (Fig. 8B). These results suggest that TMMC-induced HO-1 mediates the inhibition of TNF-α-induced ICAM-1 and COX-2 expressions and that CO or bilirubin – or both – mediate the effect of HO-1.

4. Discussion

The synthetic chalcone, TMMC, is an anti-inflammatory compound that reduces nitric oxide production by inhibiting inducible nitric oxide synthase (iNOS) gene expression through inhibition of NF-κB activation in LPS-activated RAW 264.7 cells [16]. In addition, TMMC inhibits platelet derived growth factor-induced rat hepatic stellate cells proliferation, by inhibiting the phosphatidylinositol 3-kinase-Akt-$p70^{S6K}$ pathways [17]. Here, we have shown that TMMC has a highly beneficial effect in ameliorating TNBS-induced colitis, a murine experimental model of IBD. The protective effect of TMMC was manifested by reductions in weight loss, colonic damage and mucosal ulceration. TMMC also suppressed the expression of ICAM-1, IL-1β and TNF-α in colonic mucosal tissue. Although the method used in this study was more likely to be associated with a preventive mode (pretreatment of TMMC for one day before TNBS administration), our preliminary study demonstrated that the therapeutic application of TMMC was also effective in TNBS colitis (data not shown). Acetic acid- and dextran sodium sulfate-induced colitis are the most widely used chemically induced models of intestinal inflammation [25]. Therefore, it will be interesting to determine next whether TMMC also has protective effect in other intestinal inflammatory conditions.

IECs play a pivotal role in host defense by sensing very early microbial infection, resulting in the production of a variety of proinflammatory cytokines affecting leukocyte activity [26]. Chemokines produced by IECs, such as IL-8, may provide signals essential for the initiation and maintenance of the mucosal inflammatory response. In vitro studies indicate an important role of IL-8 during acute and chronic IBD [27,28]. In addition, IBD is characterized by a high intestinal tissue turnover during the sequence of inflammation, tissue destruction and healing. MMPs appear to play an important role in the process of tissue remodeling and destruction in patients suffering from IBD [29,30]. Of the MMPs, epithelial-derived MMP-7 has been considered important in intestinal inflammation [31,32]. Moreover, MMP inhibitors ameliorate TNBS- and dextran sodium sulfate-induced colitis [33,34]. Therefore, specific inhibition of IL-8 and MMP gene expression by pharmacological modulation could have beneficial effects for treating IBD. In this study, we have shown that TMMC is an inhibitor of TNF-α-induced IL-8 and MMP-7 production in HT-29 cells.

We have reported previously that TMMC-induced HO-1 expression might be responsible for beneficial effects in macrophages and hepatic stellate cells [16,17]. Many studies have suggested that the upregulation of endogenous HO-1 expression may limit the inflammatory reaction and cell injury in colitis. Furthermore, inducing HO-1 activity may be a novel therapeutic strategy for patients with IBD [13–15]. Here, we found that TMMC also induces HO-1 expression in HT-29 cells. In addition, our studies using the HO-1 inducer hemin as well the HO-1 inhibitor SnPP suggest that TMMC-induced HO-1 gene expression might be important for its anti-inflammatory effect in TNF-α stimulated HT-29 cells. Modulation of gene transcription is the principal mechanism for the induction of HO-1 protein expression. Several pathways have been implicated in transmitting the extracellular signals to the nuclei for HO-1 gene expression. In general, HO-1 gene expression can be induced through signaling pathways such as the MAPKs [18–20]. Here, we demonstrated that HO-1 gene expression induced by TMMC was dependent on Erk1/2 and p38 activations. Thus, inhibitors of the Erk1/2 and p38 pathway significantly decreased TMMC-induced HO-1 levels.

Although the induction of HO-1 expression has been reported extensively and is known to be regulated primarily at the level of gene transcription, the molecular mechanism underlying this response is poorly understood. However, Nrf2 has been implicated in the inducer-dependent activation of the HO-1 gene [22,23]. This transcription factor is bound to different proteins in the cytoplasm and several stimuli have been shown to antagonize repression by proteins such as Keap1, leading to Nrf2 nuclear translocation and gene transcription [35]. In addition, Nrf2 nuclear translocation requires the activation of MAPK [36–38]. In our study, we found that TMMC induced Nrf2 nuclear translocation, and that Erk1/2 and p38 inhibitors blocked the TMMC-induced Nrf2 nuclear translocation. This suggests that the Erk1/2 and p38 pathways may play a role in regulating TMMC-induced Nrf2 nuclear translocation. Thus, TMMC may increase both Erk1/2 and p38 activation in HT-29 cells to stimulate Nrf2 nuclear translocation. Increased Nrf2 in the nucleus may increase its DNA-binding activity, followed by HO-1 gene transcription, eventually leading to HO-1 expression.

NF-κB regulates the transcription of a number of proinflammatory molecules involved in acute responses to injury and in chronic intestinal inflammation, including IL-1β, TNF-α, IL-6, IL-8, IL-12, iNOS, and ICAM-1. In addition, NF-κB activation, as indicated by increased DNA binding activity and p65 nuclear translocation, has been observed in the intestines of patients with Crohn's disease and ulcerative colitis, as well as in rodents with experimental colitis. In these situations the amount of activated NF-κB correlates with the degree of mucosal inflammation [39–41]. Therefore, regulation of NF-κB activation in IECs is a promising area of research in intestinal inflammatory diseases that could potentially give rise to targets for therapeutic intervention. Therefore, we investigated the relationship between HO-1 and NF-κB activation in TNF-α-stimulated HT-29 cells. Since time course analysis in HT-29 cells revealed that the onset of HO-1 expression induced by 20 μM TMMC was around 6 h, cells were incubated with TMMC for 7 h and then exposed to TNF-α. These data suggest that the TMMC-induced HO-1 protein interferes with TNF-α-induced NF-κB activation, especially the nuclear translocation step of NF-κB p65, but without affecting I-κBα phosphorylation and degradation. This is an unusual mechanism for the control of NF-κB activation. As we had found that TMMC induced both Erk1/2- and p38-dependent HO-1 gene expression in HT-29 cells (Fig. 4), we therefore tested whether specific inhibitors of Erk1/2 or p38 might alter NF-κB p65 nuclear translocation. Blocking of HO-1 expression by treatment with inhibitors reversed the suppressive effects of TMMC in terms of NF-κB p65 translocation in TNF-α-treated HT-29 cells. However, treatment with inhibitors did not influence I-κBα degradation. Thus, the TMMC-induced HO-1 protein presumably has different effects on the NF-κB activation. It has been reported that synthetic chroman and diamine compounds also specifically interfere with LPS-induced nuclear translocation of NF-κB p65 without affecting I-κB degradation, as shown for the synthetic peptide SN50 [42,43]. Although the molecular target of HO-1 has yet to be elucidated, this study is the first to show the signal pathway evoked by HO-1 during intestinal inflammation. In addition, we have also provided evidence that HO-1 acts upstream of NF-κB activity, supporting a previous study demonstrating that HO-1 expression has a direct effect on the activation of the proinflammatory NF-κB pathway [14].

Although most of the data presented in this study suggest that the protective effects of TMMC on TNF-α-induced inflammation in HT-29 cells are mediated via the induction of HO-1, the anti-inflammatory effects seen with TMMC are not related only to the expression of HO-1. This is because incubation with TMMC for 1 h, at which time HO-1 expression is not detected, also inhibited the NF-κB pathway. Therefore, we suggest that TMMC inhibits NF-κB activation both directly and indirectly through HO-1-dependent and -independent pathways in TNF-α-stimulated HT-29 cells. NF-κB activation is critical in TNF-α inducible various inflammatory molecule including COX-2 and ICAM-1. Therefore, many drugs used in the treatment of IBD have focused on the suppression of NF-κB activity [44–46]. In addition, HO-1 expression has a direct effect on the NF-κB activation. Therefore, we suggest that HO-1 induction agents may be provides important alternate target for treatment of IBD. Our data are compatible with the schematic representation in Fig. 9.

HO-1 and its metabolites, such as CO, bilirubin and iron, display several anti-inflammatory effects in vivo and in vitro. It is reported that HO-1-generated bilirubin or CO ameliorate experimental murine colitis [47–49]. Therefore, we tested whether any specific enzymatic products of HO-1 could mediate the potential protective effect in TNF-α-stimulated HT-29 cells. Both CO and bilirubin, applied independently, inhibited the expression of proinflammatory molecules, including IL-8, ICAM-1 and COX-2 as well as the production of extracellular MMP-7. Interestingly, CO and bilirubin, applied together, potentiated the inhibitory effects on TNF-α-treated

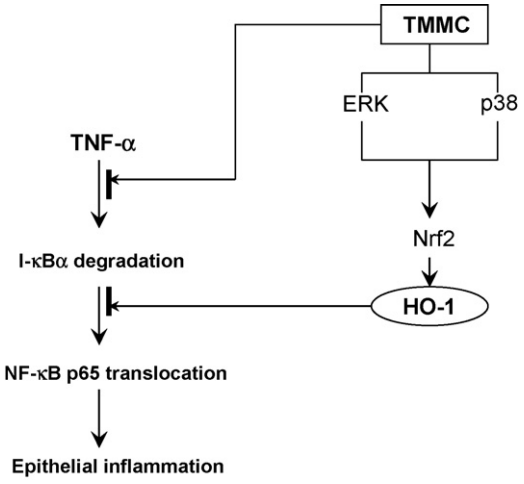

Fig. 9 – Hypothetical mechanism of action of TMMC on TNF-α-induced inflammation in HT-29 cells. TMMC might inhibit NF-κB p65 translocation directly and indirectly. The latter is mediated by HO-1, which presumably blocks the TNF-α-induced nuclear translocation of NF-κB p65 without affecting I-κBα degradation. TMMC might increase Erk1/2 and p38 phosphorylation levels to stimulate Nrf2 nuclear translocation, followed by HO-1 gene transcription, eventually leading to HO-1 expression. Sites of suppression by TMMC are indicated (⊥).

HT-29 cells. This suggests that CO and bilirubin may act in an additive or synergistic manner. Therefore, inducing HO-1 activity or treating with CO and bilirubin may be evaluated as potential tools for therapy of patients with IBD.

In conclusion, we have shown that TMMC can significantly attenuate the colonic injury and inflammation induced by TNBS in mice. TMMC induced HO-1 gene expression; a pathway involved in TMMC-induced Erk1/2 and p38 MAPK phosphorylation and this is followed by Nrf2 nuclear translocation in HT-29 cells. The inhibition of epithelial inflammation by TMMC appears to be mediated both directly and indirectly through the inhibition of the NF-κB signaling system. We also found that CO and bilirubin, enzymatic products of HO-1, mediated the potential protective effect in TNF-α-stimulated HT-29 cells.

Acknowledgment

This work was supported by the Korean Association for the Study of Intestinal Diseases, 2006 (Daewoong).

REFERENCES

[1] Farrell RJ, Peppercorn MA. Ulcerative colitis. Lancet 2002;359:331–40.
[2] Fiocchi C. Inflammatory bowel disease. Current concepts of pathogenesis and implications for therapy. Minerva Gastroenterol Dietol 2002;48:215–26.
[3] Wang WP, Guo X, Koo MW, Wong BC, Lam SK, Ye YN, et al. Protective role of heme oxygenase-1 on trinitrobenzene sulfonic acid-induced colitis in rats. Am J Physiol Gastrointest Liver Physiol 2001;281:G586–94.
[4] Talero E, Sanchez-Fidalgo S, Calvo JR, Motilva V. Chronic administration of galanin attenuates the TNBS-induced colitis in rats. Regul Pept 2007;141:96–104.
[5] Maines MD. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. FASEB J 1988;2:2557–68.
[6] Maines MD. The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol 1997;37:517–54.
[7] Morse D, Choi AM. Heme oxygenase-1: the "emerging molecule" has arrived. Am J Respir Cell Mol Biol 2002;27: 8–16.
[8] Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. Trends Immunol 2003;24:449–55.
[9] Rosenberg DW, Kappas A. Characterization of heme oxygenase in the small intestinal epithelium. Arch Biochem Biophys 1989;274:471–80.
[10] Cable JW, Cable EE, Bonkovsky HL. Induction of heme oxygenase in intestinal epithelial cells: studies in Caco-2 cell cultures. Mol Cell Biochem 1993;129:93–8.
[11] Poss KD, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. Proc Natl Acad Sci USA 1997;94:10925–30.
[12] Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, et al. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. J Clin Invest 1999;103:129–35.
[13] Tamaki T, Konoeda Y, Yasuhara M, Tanaka M, Yokota N, Hayashi T, et al. Glutamine-induced heme oxygenase-1 protects intestines and hearts from warm ischemic injury. Transplant Proc 1999;31:1018–9.
[14] Jun CD, Kim Y, Choi EY, Kim M, Park B, Youn B, et al. Gliotoxin reduces the severity of trinitrobenzene sulfonic acid-induced colitis in mice: evidence of the connection between heme oxygenase-1 and the nuclear factor-kappaB pathway in vitro and in vivo. Inflamm Bowel Dis 2006;12:619–29.
[15] Varga C, Laszlo F, Fritz P, Cavicchi M, Lamarque D, Horvath K, et al. Modulation by heme and zinc protoporphyrin of colonic heme oxygenase-1 and experimental inflammatory bowel disease in the rat. Eur J Pharmacol 2007;561:164–71.
[16] Lee SH, Seo GS, Kim JY, Jin XY, Kim HD, Sohn DH. Heme oxygenase 1 mediates anti-inflammatory effects of 2′,4′,6′-tris(methoxymethoxy) chalcone. Eur J Pharmacol 2006;532:178–86.
[17] Lee SH, Seo GS, Kim HS, Woo SW, Ko G, Sohn DH. 2′,4′,6′-Tris(methoxymethoxy) chalcone attenuates hepatic stellate cell proliferation by a heme oxygenase-dependent pathway. Biochem Pharmacol 2006;72:1322–33.
[18] Gong P, Cederbaum AI, Nieto N. Increased expression of cytochrome P450 2E1 induces heme oxygenase-1 through ERK MAPK pathway. J Biol Chem 2003;278:29693–700.
[19] Gong P, Hu B, Cederbaum AI. Diallyl sulfide induces heme oxygenase-1 through MAPK pathway. Arch Biochem Biophys 2004;432:252–60.
[20] Iles KE, Dickinson DA, Wigley AF, Welty NE, Blank V, Forman HJ. HNE increases HO-1 through activation of the ERK pathway in pulmonary epithelial cells. Free Radic Biol Med 2005;39:355–64.
[21] Khor TO, Huang MT, Kwon KH, Chan JY, Reddy BS, Kong AN. Nrf2-deficient mice have an increased susceptibility to dextran sulfate sodium-induced colitis. Cancer Res 2006;66:11580–4.
[22] Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, Cook JL. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J Biol Chem 1999;274:26071–8.
[23] Alam J, Cook JL. Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. Curr Pharm Des 2003;9:2499–511.
[24] Ma JL, Yang PY, Rui YC, Lu L, Kang H, Zhang J. Hemin modulates cytokine expressions in macrophage-derived foam cells via heme oxygenase-1 induction. J Pharmacol Sci 2007;103:261–6.
[25] Ho SB, Dvorak LA, Moor RE, Jacobson AC, Frey MR, Corredor J, et al. Cysteine-rich domains of muc3 intestinal mucin promote cell migration, inhibit apoptosis, and accelerate wound healing. Gastroenterology 2006;131:1501–17.
[26] Finlay BB, Falkow S. Common themes in microbial pathogenicity revisited. Microbiol Mol Biol Rev 1997;61: 136–69.
[27] Gerard C, Rollins BJ. Chemokines and disease. Nat Immunol 2001;2:108–15.
[28] Gijsbers K, Van Assche G, Joossens S, Struyf S, Proost P, Rutgeerts P, et al. CXCR1-binding chemokines in inflammatory bowel diseases: down-regulated IL-8/CXCL8 production by leukocytes in Crohn's disease and selective GCP-2/CXCL6 expression in inflamed intestinal tissue. Eur J Immunol 2004;34:1992–2000.
[29] Schuppan D, Hahn EG. MMPs in the gut: inflammation hits the matrix. Gut 2000;47:12–4.
[30] von Lampe B, Barthel B, Coupland SE, Riecken EO, Rosewicz S. Differential expression of matrix metalloproteinases and their tissue inhibitors in colon mucosa of patients with inflammatory bowel disease. Gut 2000;47:63–73.
[31] Salmela MT, Pender SL, Karjalainen-Lindsberg ML, Puolakkainen P, Macdonald TT, Saarialho-Kere U. Collagenase-1 (MMP-1), matrilysin-1 (MMP-7), and

stromelysin-2 (MMP-10) are expressed by migrating enterocytes during intestinal wound healing. Scand J Gastroenterol 2004;39:1095–104.
[32] Bister V, Salmela MT, Heikkila P, Anttila A, Rintala R, Isaka K, et al. Matrilysins-1 and -2 (MMP-7 and -26) and metalloelastase (MMP-12), unlike MMP-19, are up-regulated in necrotizing enterocolitis. J Pediatr Gastroenterol Nutr 2005;40:60–6.
[33] Di Sebastiano P, di Mola FF, Artese L, Rossi C, Mascetta G, Pernthaler H, et al. Beneficial effects of Batimastat (BB-94), a matrix metalloproteinase inhibitor, in rat experimental colitis. Digestion 2001;63:234–9.
[34] Naito Y, Takagi T, Kuroda M, Katada K, Ichikawa H, Kokura S, et al. An orally active matrix metalloproteinase inhibitor, ONO-4817, reduces dextran sulfate sodium-induced colitis in mice. Inflamm Res 2004;53:462–8.
[35] Nguyen T, Yang CS, Pickett CB. The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. Free Radic Biol Med 2004;37:433–41.
[36] Zipper LM, Mulcahy RT. Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of GCS genes. Biochem Biophys Res Commun 2000;278:484–92.
[37] Aburaya M, Tanaka K, Hoshino T, Tsutsumi S, Suzuki K, Makise M, et al. Heme oxygenase-1 protects gastric mucosal cells against non-steroidal anti-inflammatory drugs. J Biol Chem 2006;281:33422–3.
[38] Rushworth SA, Ogborne RM, Charalambos CA, O'Connell MA. Role of protein kinase C delta in curcumin-induced antioxidant response element-mediated gene expression in human monocytes. Biochem Biophys Res Commun 2006;341:1007–16.
[39] Lee JI, Burckart GJ. Nuclear factor kappa B: important transcription factor and therapeutic target. J Clin Pharmacol 1998;38:981–93.
[40] Jobin C, Bradham CA, Russo MP, Juma B, Narula AS, Brenner DA, et al. Curcumin blocks cytokine-mediated NF-kappa B activation and proinflammatory gene expression by inhibiting inhibitory factor I-kappa B kinase activity. J Immunol 1999;163:3474–83.
[41] Jobin C, Sartor RB. The I-kappa B/NF-kappa B system: a key determinant of mucosal inflammation and protection. Am J Physiol Cell Physiol 2000;278:C451–62.
[42] Kim BH, Reddy AM, Lee KH, Chung EY, Cho SM, Lee H, et al. Inhibitory mechanism of chroman compound on LPS-induced nitric oxide production and nuclear factor-kappaB activation. Biochem Biophys Res Commun 2004;325:223–8.
[43] Shin HM, Kim MH, Kim BH, Jung SH, Kim YS, Park HJ, et al. Inhibitory action of novel aromatic diamine compound on lipopolysaccharide-induced nuclear translocation of NF-kappaB without affecting IkappaB degradation. FEBS Lett 2004;571:50–4.
[44] Neurath MF, Pettersson S, Meyer zum Büschenfelde KH, Strober W. Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. Nat Med 1996;2:998–1004.
[45] Wahl C, Liptay S, Adler G, Schmid RM. Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. J Clin Invest 1998;101:1163–74.
[46] Maity S, Ukil A, Karmakar S, Datta N, Chaudhuri T, Vedasiromoni JR, et al. Thearubigin, the major polyphenol of black tea, ameliorates mucosal injury in trinitrobenzene sulfonic acid-induced colitis. Eur J Pharmacol 2003;470:103–12.
[47] Naito Y, Takagi T, Yoshikawa T. Heme oxygenase-1: a new therapeutic target for inflammatory bowel disease. Aliment Pharmacol Ther 2004;20:177–1784.
[48] Berberat PO, A-Rahim YI, Yamashita K, Warny MM, Csizmadia E, Robson SC, et al. Heme oxygenase-1-generated biliverdin ameliorates experimental murine colitis. Inflamm Bowel Dis 2005;11:350–9.
[49] Hegazi RA, Rao KN, Mayle A, Sepulveda AR, Otterbein LE, Plevy SE. Carbon monoxide ameliorates chronic murine colitis through a heme oxygenase 1-dependent pathway. J Exp Med 2005;202:1703–13.