Tetramethylpyrazine attenuates PPAR-γ antagonist-deteriorated oxazolone-induced colitis in mice

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Abstract. Tetramethylpyrazine (TMP) is suggested to have anti-inflammatory activity. The aim of this study was to determine the role of peroxisome proliferator activated receptor γ (PPAR-γ) signaling in the pharmacologic effect of TMP on oxazolone (OXZ)-induced colitis. TMP (80 mg/kg/day i.p.) was administered daily 48 h after intrarectal instillation of OXZ, with or without PPAR-γ inhibitor [bisphenol A diglycidyl ether (BADGE) 30 mg/kg] during the 4 days before sacrifice. The inflammatory response was assessed by the disease activity index, macroscopy, histology and myeloperoxidase (MPO) activity. Expression levels of PPAR-γ, NF-κB p65, COX-2, iNOS and TNF-α mRNA in colon mucosa were determined by FQ-PCR, levels of PPAR-γ and NF-κB p65 protein were analyzed by immunohistochemistry, and the total and phosphorylated levels of p38 MAPK were assessed by western blotting. TMP significantly attenuated the damage caused by OXZ and substantially reduced the rise in MPO activity, TNF-α, iNOS, NF-κB p65 and COX-2 expression, as well as the increase in PPAR-γ production; however, no changes in the activation of p38 MAPK were observed. Inhibition of PPAR-γ signaling aggravated inflammation of colon mucosa, and increased p38 phosphrylation. TMP counteracted the effect of inhibition of PPAR-γ. We suggest that the effect of TMP treatment in ulcerative colitis may be related to PPAR-γ signaling, but is independent of PPAR-γ.

Introduction

Ulcerative colitis (UC) is a relapsing-remitting chronic inflammatory disease of the colon that presents as diarrhea and gastrointestinal bleeding, and the incidence in China has increased over the last 20 years (1). Anti-inflammatory agents, such as sulfasalazine, various 5-aminosalicylate (5-ASA) preparations and corticosteroids, in addition to antibiotics and lifestyle modifications, have been the mainstay of UC therapy for many years. Although these therapies are often successful, some patients may remain refractory (2,3). An increased understanding of the pathogenesis of inflammatory bowel disease has led to the rapid development of other biological agents. Many of these promising agents target specific effector pathways in the inflammatory cascade, including anti-CD3 antibodies (visiluzumab), anti-IL2 receptor antibody (daclizumab), TNF-α antibody (infliximab), selective integrin blockers (MLN02) and PPAR-γ agonists (rosiglitazone) (2,3).

Tetramethylpyrazine (TMP), also known as ligustrazine, is the main active compound purified from *Ligustium wollichii franchat* (4). TMP has been used clinically for the treatment of cardiovascular disorders (5,6) and acute ischemic cerebrovascular disease. TMP was also reported to possess a very broad spectrum of pharmacological actions, such as antioxidant, anti-inflammatory (7), anti-fibrosis (8), anti-tumor and immunomodulative effects. In our previous study, it was suggested that TMP could augment the therapeutic efficiency of Salicylazosulphapyridine Salazosulfapyridine (SASP) or corticosteroids and impair experimental colitis (9). To unravel the underlying mechanism, we used cDNA array to uncover changes in the whole spectrum of gene expression after TMP treatment, and determined whether PPAR-γ was upregulated by TMP.

The peroxisome proliferator activated receptor γ (PPAR-γ) is a nuclear receptor highly expressed in the colon and plays a key role in bacterial-induced inflammation. The regulation of colon inflammation by this receptor has been well demonstrated in many experimental models of colitis and in patients with UC, which is characterized by impaired expression of PPAR-γ confined to colon epithelial cells (10). To determine whether the anti-inflammatory effects of TMP are through the PPAR-γ pathway, we used bisphenol A diglycidyl ether (BADGE) to block PPAR-γ and compared the therapeutic effect of TMP with and without PPAR-γ inhibition.

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Table I. Effect of TMP and/or BADGE on clinical parameters of oxazolone-induced colitis in mice (mean ± SD).

Group	DAI	Macroscopy score	Histology score	MPO activity (U/g)
NC	0.60±0.55	1.00±0.71	1.00±0.71	0.25±0.03
OXZ	8.80±0.45[a]	6.60±0.55[a]	14.00±1.22[a]	0.50±0.04[a]
TMP	4.60±0.55[a,c]	3.60±0.55[a,c]	4.60±1.52[a,c]	0.34±0.02[a,c]
BA	8.80±0.45[a]	7.20±0.84[a]	17.8±0.84[a,c]	0.56±0.04[a]
TMP and BA	4.80±0.45[a,e]	3.80±0.45[a,e]	7.40±0.89[a,e]	0.53±0.05[a,f]

[a]$P<0.01$, compared to the NC group; [b]$P<0.05$ and [c]$P<0.01$, compared to the OXZ group; [d]$P<0.05$ and [e]$P<0.01$, compared to the BA group; [f]$P<0.05$ and [g]$P<0.01$, compared to the TMP group. DAI, disease activity index.

Materials and methods

Mice and induction of colitis. Male, 7-8-week-old Kunming (KM) mice were obtained from the Experimental Animal Center of Shanghai Fudan University (Shanghai, China). The mice were kept under standard laboratory conditions, and had free access to food and water for at least 1 week before the experimental period. Colitis was induced by OXZ, (Sigma Chemical Company, St. Louis, MO, USA) as previously described by Heller *et al* (11). In brief, mice were pre-sensitized with OXZ dissolved in 100% ethanol (3% wt/vol) by application to the skin of the abdomen. Five days after pre-sensitization, mice were re-challenged intrarectally with 150 Ul 1% OXZ in 50% ethanol or 50% ethanol only.

Treatment protocols. Fifty mice were randomly allocated into the following groups: i) normal control group, mice received 0.15 ml 50% ethanol intrarectally instead of 1% OXZ in 50% ethanol (n=10); ii) OXZ group, 100 μl of 0.9% saline solution was administered by intraperitoneal injection 24 h after OXZ instillation and then daily during the 4 days before the animals were sacrificed; iii) TMP group, 80 mg/kg TMP (The Seventh Wuxi Pharmaceutical Factory, Jiangsu, China; batch no. 06062013, diluted in 0.9% saline) was administered intraperitoneally every day starting 24 h after colitis induction for 4 days (n=10); iv) BA group, instead of TMP, BADGE was administered [30 mg/kg intraperitoneally (Sigma Chemical Co.) in 10% DMSO]; v) TMP and BA group, BADGE and TMP combination, where 30 mg/kg BADGE was administered intraperitoneally 30 min prior to TMP (N=10). After 4 days of TMP and/or BADGE treatment, all mice were sacrificed and analyzed.

Clinical activity score of colitis disease activity index (DAI). Analyses of the clinical severity of colitis including change in body weight (BW), as well as stool consistency and rectal bleeding were performed daily using a scoring system described previously (Hartmann *et al*). In brief, the loss of BW was scored as follows: 0, no weight loss; 1, weight loss of 1-5%; 2, weight loss of >5-10%; 3, loss of >10-20%; and 4, weight loss >20%. Assessment of diarrhea (stool consistency) was as follows: 0, normally formed pellets; 2, pasty and semi-formed pellets; and 4, liquid stools. Bleeding was scored as follows: 0, no blood in hemocult; 2, positive hemocult; and 4, gross bleeding from the rectum. The resulting scoring parameters were added together to obtain a total DAI ranging from 0 (healthy) to 12 (maximal ill/activity of colitis).

Macroscopic score and histological score. The distal colon was removed, opened longitudinally and the mucosal damage was scored on a 0-10 scale according to the criteria of Bobin-Dubigeon *et al* (12). Histological examination was performed using a sample of colon tissue located precisely 3 cm above the anal canal in mice from all treatment groups. The colon tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin for histological analysis. Four micrometer sections were deparaffinized with xylene and stained with hematoxylin and eosin (H&E) using routine techniques. Tissues were scored semi-quantitatively from 0 to 5 (0, no changes to 5, marked transmural inflammation with severe ulceration and loss of intestinal glands) in a blinded fashion according to previously described criteria (13,14).

Myeloperoxidase activity (MPO). MPO activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as previously described (15). Tissue samples were homogenized in HTAB (0.5% in distilled water) and the homogenates were centrifuged at 10,000 x g for 20 min at 4°C and the clear supernatant was used for the assay. The substrate O-dianisidine dihydrochloride (16.7 mg) was dissolved in 90 ml of distilled water followed by 10 ml of potassium phosphate buffer (pH 6.0) and 50 μl of freshly prepared hydrogen peroxide (H_2O_2, 1%). An aliquot of 14 μl of each sample was used for the micro-titer plate assay, and 200 μl of O-dianisidine dihydrochloride solution was added to each well immediately prior to reading the plate. The resultant oxygen radical (O^-) from the interaction between MPO in the sample and H_2O_2, combined with O-dianisidine dihydrochloride, the hydrogen donor (AH_2), converted it into a colored compound. The color development was measured at an absorbance of 460 nm over a period of 5 min. MPO activity of the tissue samples was calculated based on the change in absorbance/min. The change in absorbance for 1 μmol H_2O_2 split was 1.13×10^{-2} and the MPO content of the samples was calculated based on the formula; 1 unit of MPO =1 μmol H_2O_2 split.

Real-time PCR. Total-RNA from murine colon specimens was extracted using the TRIzol extraction method (Invitrogen Life Technologies, Carlsbad, Ca). Total-RNA (1 μg) was reverse transcribed using an oligo(dt) primer. Real-time PCR

Table II. mRNA level of PPAR-γ, NF-κB p65, COX-2, iNOS, TNF-α in colonic mucosa in all groups (mean ± SD).

	mRNA (%)				
	PPAR-γ	NF-κB p65	COX-2	iNOS	TNF-α
NC	0.41±0.03	0.21±0.02	0.43±0.14	0.32±0.13	0.02±0.01
OXZ	0.119±0.014[b]	0.548±0.026[b]	1.934±0.506[b]	0.834±0.062[b]	0.049±0.009[b]
TMP	0.262±0.028[b,d]	0.258±0.028[d]	0.645±0.287[d]	0.290±0.122[d]	0.027±0.006[d]
BA	0.218±0.087[b]	1.076±0.228[b,d]	1.552±0.234[b]	1.084±0.274[b,c]	0.076±0.023[b,d]
TMP and BA	0.224±0.088[b]	0.715±0.304[b]	0.888±0.262[a,d,f]	0.535±0.174[f,d]	0.040±0.018[f]

[b]P<0.01, compared to the NC group; [c]P<0.05 and [d]P<0.01, compared to the OXZ group; [e]P<0.05 and [f]P<0.01, compared to the BA group; [g]P<0.05 and [h]P<0.01, compared to the TMP group.
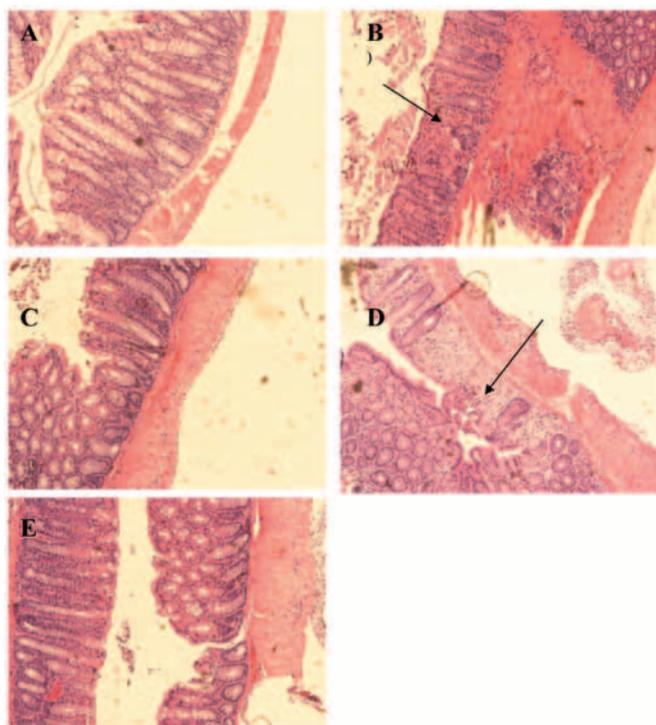

Figure 1. H&E staining of colonic mucosa. (A) Ethanol-treated control, (B) oxazolone-treated group, (C) oxazolone-treated + TMP 80 mg/kg group, (D) oxazolone-treated + BADGE 30 mg/kg group and (E) oxazolone-treated + TMP 80 mg/kg + BADGE 30 mg/kg group. There was marked typical colitis in the OXZ group and oxazolone-treated + BADGE group.

analysis for quantification of the expression of TNF-α mRNA was performed in duplicate on an ABI-7300 Real-Time PCR System (Applied Biosystems) using the ABsolute SYBR-Green Fluorescein kit (Abgene, Thermo Scientific). QuantiTect primer sets were used for these experiments (Qiagen). TNF-α, sense CTCACCCACACCGTCAGCCGATTT, and anti-sense TaqMan-probe, FAM-TGCAGCAGCATGTTCTGGAAGC-TAMRA were used.

Western blotting. Tissue samples were collected from all the mice. The samples were homogenized in Tris-HCl buffer with a cocktail of protease inhibitors and then centrifuged. Supernatants were subjected to SDS-PAGE electrophoresis (20 μg protein per sample/lane) and electrophoretically transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% dried non-fat milk in PBS buffer and 0.05% Tween-20. The membranes were then incubated with primary antibodies against p38 MAPK (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:250 and phosphorylated p38 MAPK (Santa Cruz Biotechnology), diluted 1:200; membranes were then washed with PBS-T buffer (PBS with 0.1% Tween-20) and incubated with the appropriate secondary antibodies. Immunoreactive proteins were visualized with enhanced chemiluminescence (Pierce Biotechnology, USA).

Immunohistochemical assay. Deparaffined sections were incubated with primary antibodies as follows: rabbit anti-p65 and PPAR-γ (Santa Cruz Biotechnology). Sections were incubated with isotype control serum alone instead of primary antibody as a negative control, or with the corresponding blocking peptide to identify the specificity of the antibodies. After a 2-h incubation at room temperature, sections were washed with PBS. The Enhanced Labeled Polymer System (EnVision, Dako, Denmark) was added and incubated for 30 min at room temperature. Hematoxylin was used for nuclear counterstaining after peroxidase (DAB) development. Images were captured using an Olympus BH2 microscope and IHC image analysis software (Shanghai Shenteng Information Technology Ltd.).

Statistical analysis. All data are expressed as the mean ± SD. Statistical significance among the groups was evaluated by one-way analysis of variance (ANOVA). The Mann-Whitney U test was chosen for non-parametric values. P-values of <0.05 were considered statistically significant.

Results

Animals treated with OXZ rapidly developed colitis marked by weight loss, diarrhea, and a high DAI (Table I). In accordance with these observations, histologic examination revealed loss of normal architecture, a mixed cell inflammatory infiltrate, and areas of epithelial erosion, along with increases in MPO and TNF-α expression (Table I and II, Fig. 1B). Control mice treated with 45% ethanol alone did not develop wasting disease
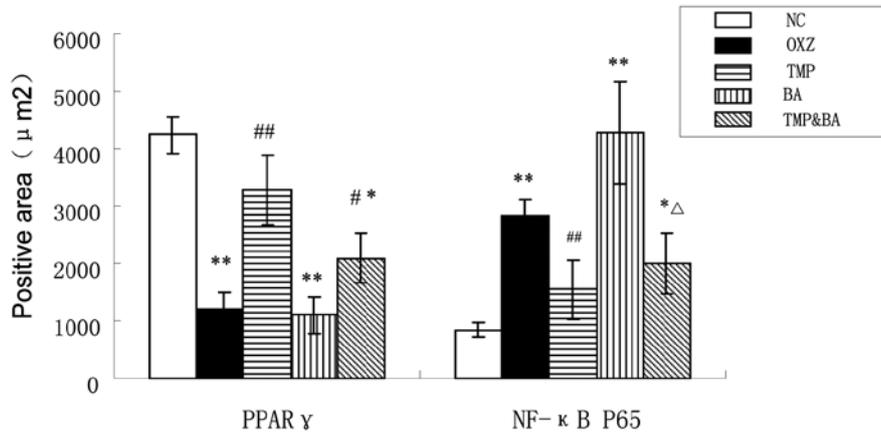
Figure 2. Quantified positive staining area of PPAR-γ, NF-κB p65 in colonic mucosa of all groups by immunohistochemical staining. Mouse colitis was induced by oxazolone or treated with ethanol as control (NC). Colitis mice were treated with vehicle control (OXZ) or BADGE 30 mg/kg (BA) or TMP 80 mg/kg (TMP) or BADGE 30 mg/kg and TMP 80 mg/kg (BA and TMP). *P<0.05 and **P<0.01, compared to the NC group; #P<0.05 and ##P<0.01, compared to the OXZ group, △P<0.01, compared to the TMP group.

A
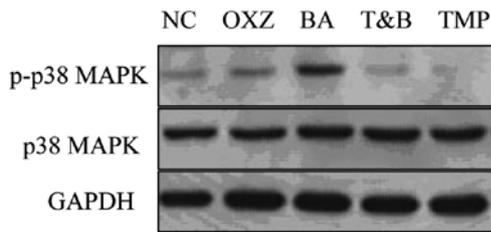
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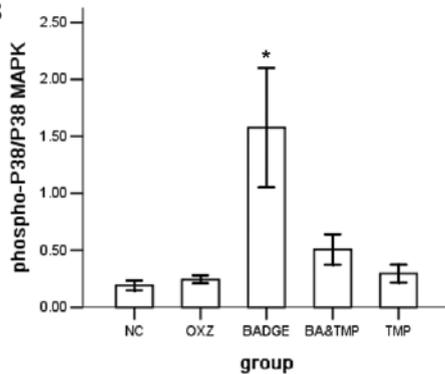
Figure 3. Western blot analysis of the expression and phosphorylation of p38 MAPK. (B) Densitometry evaluation of the ratio of phosphor-p38 to p38. The bars represent the mean ± SEM. Mouse colitis was induced by oxazolone or treated with ethanol as control (NC). Colitis mice were treated with vehicle control (OXZ) or BADGE 30 mg/kg (BA) or TMP 80 mg/kg (TMP) or BADGE 30 mg/kg and TMP 80 mg/kg (BA and TMP). *P<0.05, BADGE vs. each of the other 4 groups.

and exhibited a healthy appearance. Treatment with TMP resulted in a significant reduction in clinical, macroscopic and microscopic parameters of colitis, and decreased MPO activity and TNF-α expression (Table I and II, Fig. 1C).

Inhibition of PPAR-γ by BADGE significantly increased the histological disease score and TNF-α compared with the group treated with OXZ alone, but not DAI, macroscopic parameters and MPO activity. Co-administration of BADGE

and TMP did not abolish attenuation of colitis by TMP, and the DAI in the BADGE and TMP group was statistically similar to that in the TMP group. Macroscopic parameters, histological score, and TNF-α in the TMP treatment group were increased by inhibition of PPAR-γ (TMP group vs. BADGE and TMP group). When the PPAR-γ pathway was blocked, TMP could still impair colitis. Co-administration of BADGE and TMP reduced DAI, macroscopic and histologic scores by 48, 47 and 58%, separately, compared to the BADGE group, which were similar to those in the absence of BADGE (Table I and II, Fig. 1C).

PPAR-γ was reduced in OXZ-induced colitis mucosa, compared to the normal control group, whereas TMP treatment restored PPAR-γ expression in both the PPAR inhibited and non-inhibited conditions. Administration of BADGE did not decrease the PPAR-γ level (TMP vs. TMP and BADGE, BADGE vs. OXZ) (Fig. 2).

Considering that MAPK and NF-κB are the two major downstream targets of PPAR, we determined these two parameters in each group. Consistent with the severity of the colitis pattern in each group, NF-κB p65 was overexpressed and translocated to the nucleus in OXZ-induced colitis, and was reduced by TMP treatment. BADGE alone increased the NF-κB p65 level further, whereas in the BADGE+TMP group and the BADGE group, NF-κB p65 was also reduced by TMP without PPAR-γ activity (Fig. 2).

To determine p38 MAPK pathway activation, we used western blotting to analyze p38 phosphorylation. p38 was not activated in OXZ-induced colitis, while inhibition of PPAR by BADGE dramatically increased p38 MAPK phosphorylation. This activation was reversed to normal levels by TMP (Fig. 3). These results indicate that TMP can downregulate the NF-κB and the MAPK pathway independent of PPAR-γ.

Discussion

This study indicated that TMP can attenuate OXZ-induced colitis through NF-κB and the MAPK pathway, which is PPAR-γ independent. First, TMP does not only reduce the

clinical score of OXZ-induced colitis, but is also an inflammatory cytokine. PPAR-γ was unregulated by TMP; however, the expression of PPAR-γ did not contribute significantly to the effects of TMP, as inhibition of PPAR-γ by BADGE did not abrogate the attenuation of colitis by TMP. In contrast, TMP inhibited PPAR-γ downstream pathways, both NF-κB and MAPK. Interestingly, MAPK, which was activated by blocking PPAR-γ, was restored to normal by the administration of TMP. NF-κB p65 was overexpressed and translocated to the nucleus in the colitis and BADGE group, which was also reversed by TMP.

Although Chuanxiong has been used in China for thousands of years, TMP as the main active compound has been unknown until now. Recently, the pharmacological effects of TMP have been expanded from its well-known vasodilatory actions and antiplatelet activity to a wide variety of activities such as anti-inflammatory, anti-stress [tetramethylpyrazine protects against palmitate-induced oxidative damage and mitochondrial dysfunction in C2C12 myotubes (16)], and anti-apoptosis activity. TMP can protect against MNU-induced retinal damage (17), iron-induced oxidative damage and apoptosis in cerebellar granule cells (18), gastric mucosal injury in a rat model of acute necrotizing pancreatitis, spinal cord ischemia, gentamicin-induced apoptosis in murine renal tubular cells (19), rat asthma, acute liver and lung injury after burn trauma (20), acute myocardium injury after thermal trauma (21) and accelerated anti-glomerular basement membrane antibody nephritis (22). Several studies have also reported that one of the underlying mechanisms of TMP is its reactive oxygen species (ROS)-scavenging activity (21-24). ROS are also important mediators in the pathogenesis of inflammatory bowel disease (IBD). iNOS was overexpressed and ROS-producing cells were increased in the lamina propria of IBD patients (25). Antioxidants provided protection against DSS-induced colitis (26). Whether anti-OXZ-colitis activity is mediated by ROS-scavenging requires further investigation.

PPAR-γ belongs to the nuclear receptor family. Studies have reported the therapeutic effects of ligands such as PPAR-γ in various animal models of colitis induced in different ways, including chemical compounds (27,28), bacteria (29), ischemia-reperfusion (30) and in chronic colitis occurring after the transfer of immunocompetent T cells in SCID mice (31) or spontaneously in IL-10-deficient mice (32) and in SAMP1/YitFc animals (33). Impaired expression of PPAR-γ was found in colon epithelial cells of UC patients. A randomized placebo-controlled trial showed that rosiglitazone was efficacious in the treatment of mild to moderately active UC. Our data show that PPAR-γ is decreased in colitis mucosa, and blocking PPAR-γ can further increase the histological score of OXZ-induced colitis, which may confirm the important role of PPAR-γ. The impaired expression of PPAR-γ can be restored by TMP, which may indicate that TMP can attenuate colitis through PPAR-γ. Thus, we wondered whether a PPAR-γ inhibitor could block the therapeutic effect of TMP. Unexpectedly, BADGE did not block the effect of TMP, in contrast, TMP restored BADGE-induced exacerbation of colitis in some respects.

Upon activation, PPAR-γ binds in a heterodimeric fashion with retinol X receptor-α (RXRα) to peroxisome proliferator responsive elements (PPREs) which are present on a number of gene promoter regions (10) and inhibit its transcription. Many of these PPREs are found on promoters associated with pro-inflammatory cytokines, such as TNFα and IL-1, which are inhibited by PPAR-γ (10). In addition to directly regulating cytokine production, PPAR-γ agonists may indirectly affect pro-inflammatory genes by inhibiting p38 MAPK pathways and the NF-κB pathway (34). It has been demonstrated that these two pathways play a critical role in the regulation of inflammation. NF-κB was identified as one of the key regulators of inflammation in IBD (35). Its activation is markedly induced in IBD patients through its ability to promote the expression of various pro-inflammatory genes. 2,4,6-Trinitrobenzene sulfonic acid (TNBS)-induced colitis can be successfully treated by local administration of p65 antisense oligonucleotides (36). Our results also showed that mRNA and nuclear protein expression of NF-κB p65 was significantly higher in the OXZ-induced colitis group than normal controls, while TMP decreased NF-κB p65 levels (Table II). As expected, BADGE increased the NF-κB p65 level further, which was reversed by TMP. This may indicate that TMP can inhibit the NF-κB p65 pathway in a PPAR-γ independent manner.

There are four major groups of MAPKs in mammalian cells, the extracellular signal-related kinases (ERKs), the c-Jun NH2-terminal kinases (JNKs), p38 MAPK and ERK5/big MAPK (37), which are activated by phosphorylation. MAPK cascade transducer signals from the cell surface change the gene expression that controls diverse functions such as the inflammatory response of epithelial cells (38), proliferation, differentiation, transformation and apoptosis (39). In particular, p38 MAPK is a key modulator of several target genes that ultimately control infiltration of monocytic cells, acute intestinal inflammation and intestinal electrolyte and water secretion, and in response to a variety of stimuli regulates cytokine production (40). Nevertheless, the role of p38 MAPK in IBD is controversial. An early study reported that p38 was activated and linked to TNF-α production in IBD. However, a later study showed that the activity of p38 was similar in IBD patients and controls and not modified by inflammation (41). Administration of the p38 MAPK inhibitor, SB203580, was ineffective in the treatment of TNBS-induced colitis (41), but was able to ameliorate DSS-induced colitis (42). Our results indicate that the expression and activity of p38 were similar in the OXZ-induced experimental mouse model of colitis and in controls, which was also found in a previous study. The PPAR-γ antagonist, BADGE, dramatically increased p38 activity, which may have partially led to an increase in several parameters of colitis severity. Interestingly, TMP abolished the activation of p38 by BADGE, and caused colitis amelioration. This indicates that p38 serves a different role in different models or stages of IBD development.

In conclusion, TMP reduces the development of experimental colitis and alleviates the inflammatory response. We suggest that inhibition of p38 MAPK signaling and decreased NF-κB p65 expression by TMP, independent of PPAR-γ activity, could be one of the mechanisms involved in this process.

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