ORIGINAL RESEARCH

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Tetramethylpyrazine improves oxazolone-induced colitis by inhibiting the NF- κ B pathway

Abstract

Purpose: Tetramethylpyrazine (TMP) is an effective Chinese plant-derived medicine for colitis in the clinic, but the underlying molecular mechanisms of its use remain poorly understood. The purpose of this study was to investigate the mechanisms involved in its therapeutic action.

Methods: A colitis mouse model was induced by oxazolone enema. TMP was administered at 80 mg/kg/day and sulphasalazine (SASP) was used as positive control and administered at 100 mg/kg/day for the treatment of colitis. On the fourth day after enema, mice were sacrificed. The inflammatory response was assessed by the disease activity index and histology. Colon mucosa was isolated and biochemically analyzed. In addition, *in vitro* studies were performed to evaluate the activity of TMP in Caco-2 cells.

Results: Our results showed that TMP improved the colonic inflammatory status as evidenced by histological findings, as well as SASP. These effects were associated with a decrease in nucleus translocation of NF- κ B. Paired with this inhibitive activity, there was a decrease in downstream signaling, such as C-MYC, iNOS and COX-2. *In vitro* assays revealed that TMP inhibited NF- κ B translocation and its downstream production of inflammatory factors, such as TNF- α , IL-6 and IL-8, and that ROS production that was induced by LPS in Caco-2 cells.

Conclusion: TMP improved the colitis induced by oxazolone, and its activity was associated with inhibition of NF- κ B translocation, and subsequent inhibition of pro-inflammatory factor production and oxidative stress.

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Ulcerative colitis (UC) and Crohn's disease (CD) are chronic human inflammatory bowel diseases (IBD), of which etiology and pathogenesis remain largely unknown. Previous studies showed that IBD is associated with genetic alterations [1] and environmental factors [2-3]. It is characterized by the destruction of the gastrointestinal tract integrity, the excessive production of cytokines and the subsequent chronic intestinal inflammation. Conventional therapies for IBD focus on suppression and control of inflammation by using 5-aminosalicylates, corticosteroids and immune-modulating drugs, but none of these has been found to be totally effective [4-6]. While these drugs exhibit beneficial effects on the treatment of IBD, they may impose risk to the patients. Development of new agents that combine pharmacologic efficacy and the absence of undesirable adverse effects are critical. Tetramethylpyrazine (TMP), a purified and chemically identified component of *Ligusticum wallichii* or *Ligusticum chuanxiong*, used in Chinese traditional medicine, has been shown to prevent lipid peroxidation by scavenging oxygen free radicals. The use of TMP has been an innovative approach for the treatment of IBD. A previous study demonstrated that TMP can be used in UC treatment [7], but the mechanism underlying its therapeutic action has still not been fully elucidated.

The intestinal epithelium has two important functions: it acts as a physical barrier separating luminal bacteria and immune cells and as an immunological barrier producing antimicrobial peptides and cytokines. Disruption of epithelial integrity by dysregulation of the NF- κ B pathway causes spontaneous chronic inflammation because of translocation of bacteria into mucosa [8]. It has also been reported that the nuclear factor, NF- κ B, plays an essential part in the regulation of a large number of genes involved in the immune and inflammatory responses [9-10]; therefore, the expression of NF- κ B plays an important role in the pathogenesis of IBD. Under normal situations, the intestinal mucosa is in a state of 'controlled' inflammation regulated by a delicate balance of proinflammatory (tumour necrosis factor (TNF- α), IL-1, IL-6 and IL-12) and anti-inflammatory cytokines (IL-4 and IL-10). The mucosal immune system is the central effector of intestinal inflammation and injury, with cytokines playing a central role in modulating inflammation. Cytokines may, therefore, be a logical target for IBD therapy using specific cytokine inhibitors. Agents targeted against TNF α , T-helper cell (Th-1) activation or NF- κ B, and other miscellaneous therapies, are being evaluated as potential therapies for IBD [11]. Infliximab, a monoclonal antibody of TNF α , has emerged as an efficient treatment with many clinical benefits such as quick disease activity reduction and IBD patient life quality improvement [12]. Reactive oxy-

gen species (ROS) are also involved in the development of tissue injury in patients with IBD. Actually, the anti-inflammatory effects of corticosteroids, used in the treatment of IBD, have been partly ascribed to ameliorating oxidative insult [13], and sulphasalazine has been suggested to produce beneficial effects by inhibiting the formation of ROS from neutrophils [14].

In this study, we investigated the activity of NF- κ B in oxazolone-induced colitis in mice, which is an experimental colitis that has a histologic resemblance to human ulcerative colitis, and the effect of tetramethylpyrazine on the activity of NF- κ B. The results showed that TMP inhibited the NF- κ B pathway, pro-inflammatory cytokine production and oxidative stress, which have important implications for the understanding and treatment of human ulcerative colitis.

Methods

Materials

Oxazolone was purchased from Sigma Chemical Company (St. Louis, MO, USA). Tetramethylpyrazine was produced by Wuxi Seventh Pharmaceutical Factory of China (Jiangsu, China). NF- κ B P65 and TBP antibodies were purchased from Abcam (Cambridge, UK). C-MYC, iNOS, COX-2 and β -actin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit antibody was purchased from Pierce (Rockford, IL, USA). TNF- α , IL-6, IL-8 and INF- γ ELISA Kit were purchased from R&D Systems (Minneapolis, MN, USA).

Animals

Seven-week-old male KM mice (25–30 g) were bred and maintained under specific pathogen-free conditions in the Experimental Animal Center of Shanghai Jiao Tong University. Mice were housed under standard environmental conditions (21°C, 60–70% humidity) with 12 h light/dark cycles and air filtration. All animal procedures were approved by the Animal Care Committees of Shanghai Jiao Tong University.

Induction of experimental colitis

The method of inducing colitis has been previously described by Heller *et al.* [15]. Forty-eight mice were randomized into six groups (n=8): non-colitic control group (N), oxazolone-induced colitic group (Mod), saline-treated colitic group (NS), tetramethylpyrazine-treated group (80 mg/kg/day i.p.), SASP-treated group (100 mg/kg/day i.p.), TMP and SASP co-administered group. All mice were treated with oxazolone, ex-

cept the normal control group. A piece of back skin with an area of 2cm $\times$ 2cm was shaved in advance. Mice were sensitized by skin painting with 0.2 ml 3% oxazolone in 100% ethanol on two successive days. Then five days later the mice were challenged with the intrarectal administration of 0.15 ml 0.5% oxazolone in 50% ethanol. The disease activity index (DAI), which incorporates weight, stool consistency and hematochezia, was monitored daily (Supplementary Table 1). All mice were sacrificed on the fourth day after enema. Representative whole gut specimens were taken from a region of the inflamed colon corresponding to the segment adjacent to the gross macroscopic damage, and stained with hematoxylin and eosin. Stained sections were examined for evidence of colitis using a validated scoring system [16].

Preparation of colonic epithelial cells and its lysate

Colonic epithelial cells were prepared as previously described [17]. The dissected colon was opened by cutting longitudinally and gently rinsed with ice-cold phosphate-buffered saline (PBS). Fresh digestive enzymes were added for digestion, and the sample was shaken for 30 min at 37°C. The cell suspension was collected by centrifugation, and this step was repeated five times. The cells were then suspended in 33% Percoll and centrifuged for 20 min at 400 g. After centrifugation, the cells on the top of the 33% Percoll were collected and used as epithelial cells. Then, cytosol and nuclear proteins were separated by Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, USA). The proteins were extracted according to the manufactures protocol.

Western blot

Proteins separated by gel electrophoresis were transferred to PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked with Tris-buffered saline (TBS-0.05% Tween 20) containing 3% bovine serum albumin. After incubation with the appropriate primary antibody (NF- κ B P65, COX-2, iNOS or C-MYC), bound antibody was detected by using peroxidase-conjugated secondary antibody and ECL Advance Western Blotting Detection Kit (GE Healthcare UK Ltd., Buckinghamshire, England).

Cell culture and treatment

The human colon adenocarcinoma cell line, Caco-2, was obtained from the American Type Culture Collection (ATCC). Cells were grown in DMEM supplemented with 10% FBS and 2 mM L-glutamine in a humidified 5% CO₂ atmosphere at 37°C. Caco-2 cells were seeded onto 6-well plates at a density

of 2 $\times$ 10⁵ cells per well and grown until formation of a monolayer. The cells were pretreated with TMP (40 μ g/ml) for 24 h, and stimulated with lipopolysaccharide (LPS) for 6 h at a concentration of 100 ng/ml. The supernatants were collected, centrifuged at 10,000g for 5 min, and frozen until ELISA analysis of TNF- α , IL-6 and IL-8 concentration. Cytoplasm and nucleus proteins were then separated by Nuclear and Cytoplasmic Extraction Reagents for Western blot analysis of NF- κ B P65 expression.

CD4 effectors were generated as previously reported [18]. Purified naive CD4⁺ cells (3 $\times$ 10⁵/ml) were stimulated with IL-2 (10 μ g/ml) and anti-IL-4 (10 μ g/ml) for polarization to Th1 effectors in RPMI medium, supplemented with penicillin (200 μ g/L), streptomycin (200 μ g/L), and glutamine (4 μ M) plus 10% selected FCS from Hyclone Labs (Logan, UT, USA). After 5 d, Th1 effectors were harvested and seeded onto 6-well plates at a density of 2 $\times$ 10⁵ cells per well. The cells were pretreated with TMP (40 μ g/ml) for 24 h, and stimulated with lipopolysaccharide (LPS) for 6 h at a concentration of 100 ng/ml. The supernatants were collected, centrifuged at 10,000g for 5 min, and frozen until ELISA analysis of INF- γ concentration. Cytoplasm and nucleus proteins were then separated by Nuclear and Cytoplasmic Extraction Reagents for Western blot analysis of INF- γ and P65 expression.

Measurement of ROS production

Caco-2 cells were loaded with 2 μ M 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) in serum-free low glucose-DMEM at 37 °C for 30 min and then washed twice with phosphate buffered saline (PBS). H2DCF-DA fluorescence was monitored with a flow cytometer (BD Bioscience, city, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. ROS was determined by comparing the changes in fluorescence intensity with that of the baseline (F/F₀).

Statistical analysis

All results are expressed as the mean $\pm$ S.E.M., and the differences between means were tested for statistical significance using one-way analysis of variance (ANOVA). Statistical significance was set at $p < 0.05$.

Results

TMP improves oxazolone-induced colitis in mice

Mice were rapidly induced colitis by the administration of oxazolone marked by weight loss and diarrhea with or without hematochezia 24h after challenge. Especially, this phenomenon

FIGURE 1. TMP alleviated the oxazolone-induced colitis. Mouse colitis was induced by the administration of oxazolone, representative specimens were taken from a region of colon, and full-thickness sections of 6 μ m of colon mucosa were obtained and stained with hematoxylin and eosin for histological examination. Oxazolone-induced colitis model group showed colon wall was inflamed with fibrotic thickening of tissue. The colitis model also exhibited extensive ulceration. TMP showed an anti-inflammatory effect on colitis, similar to the effects of SASP, as demonstrated by a decrease in ulcers of colonic mucosa. A) non-colitic group, B) oxazolone-induced colitic group, C) saline-treated colitic group, D) tetramethylpyrazine treated group, E) SASP treated group, and F) TMP and SASP co-administrated group. The magnification is 40 $\times$.

was most severe on the third day in the model group. The disease activity index (DAI) score, a macroscopic score to assess colitis, showed that colitic model group was significantly higher than that of non-colitic group over 4 days (s 1). TMP, as well as SASP, significantly improved the status of mice colitis and the co-administration of TMP and SASP had a synergistic effect on disease.

On histological examination, the histological features of the colon from normal group mice were typical of a normal structure (Figure 1A). Oxazolone-induced colitis model group showed inflamed colon walls with fibrotic thickening of tissue,

TABLE 1. Disease activity index (DAI) and histology scores

Group	DAI	Histology scores
N	0.19 $\pm$ 0.12	0.45 $\pm$ 0.34
Mod	3.52 $\pm$ 0.31*	12.25 $\pm$ 3.13*
NS	3.43 $\pm$ 0.17*	12.16 $\pm$ 3.65*
TMP	1.85 $\pm$ 0.14 [†]	6.46 $\pm$ 1.28 [†]
SASP	2.04 $\pm$ 0.21 [†]	6.74 $\pm$ 1.12 [†]
TMP+SASP	1.21 $\pm$ 0.12 [†] &	2.94 $\pm$ 0.78 [†] &

N: non-colitic group; Mod: oxazolone induced colitis group;

NS: saline treated group;

TMP: tetramethylpyrazine treated group;

SASP: sulphasalazine treated group.

* $P < 0.05$ compared with N group,

[†] $P < 0.05$ compared with either Mod or NS group,

& $P < 0.05$ compared with either TMP or SASP group,

Data are presented as (mean $\pm$ SD)

extensive ulceration and inflammation involving all the intestinal layers of the colon (Figure 1B). These samples were also characterized by severe edema and interstitial micro-hemorrhages. The macroscopic analysis of the colonic specimens in the colitic rats revealed existence of severe necrosis and inflammation of the mucosa.

TMP showed an anti-inflammatory effect in colitis mice, similar to the effects obtained with SASP, as demonstrated by the decreased ulcers of colonic mucosa (Figure 1D, E), but not in saline group (Figure 1C). Thus, the histological studies showed that treatment with TMP or SASP promoted recovery of the intestinal cytoarchitecture, the reduction of ulceration and edema, and depletion of mucus in the submucosal layers. In addition, co-administration of TMP and SASP produced a much stronger protective effect (Figure 1F). Briefly, the histological scores in colitis groups was significantly higher than that in normal control group, and TMP significantly improved the histological features, as well as SASP. And the co-administration of TMP and SASP had a synergistic effect (Table 1).

TMP inhibits activation of NF- κ B in colonic epithelial cells

To investigate the molecular mechanisms, epithelial cells were isolated from the colon samples. The cytoplasm and nucleus proteins were extracted separately. In the colitis model group, NF- κ B P65 was translocated to the nucleus as shown by a decrease in cytoplasmic and an increase in nuclear protein levels, indicating the activation of NF- κ B. As shown in Figure 2, TMP showed a blocking effect on oxazolone-induced NF- κ B translocation into nucleus of colonic epithelial cells, as demon-

FIGURE 2. TMP inhibited the nucleus translocation of NF- κ B P65 in colonic epithelial cells. **A)** Western blot analysis of NF- κ B level in cytosol and nuclear extract. **B)** Gray scale intensity of bands were analyzed by Image J. * $P < 0.05$ compared with N group, $^{\#}P < 0.05$ compared with either Mod or NS group, $^{\&}P < 0.05$ compared with either TMP or SASP group

strated by the decreased cytoplasmic p-IKBA and increased cytoplasmic NF- κ B p65 protein levels, which lead to the decreased nuclear NF- κ B p65 protein levels. Similar results were observed in SASP treatment group. In addition, the co-administration of TMP with SASP showed a synergistic effect in repression of nucleus transportation of NF- κ B P65.

TMP inhibits the expression of NF- κ B downstream pathway

As is well known, the nucleus translocation of NF- κ B induces the transcription of a number of inflammation and cell proliferation-related genes. Among them, iNOS, COX-2 and C-MYC are well-known downstream proteins associated with the activation of NF- κ B. Thus, the expression of these proteins was assessed by Western blot analyses. As shown in Figure 3, the expression of C-MYC, iNOS and COX-2 were significantly elevated in colitis model group, as compared with the

FIGURE 3. TMP inhibited the expression of NF- κ B downstream pathway. **A)** Western blot analysis of c-MYC, iNOS and COX2 level in cytosol and nuclear extract. **B)** Gray scale intensity of bands were analyzed by Image J. * $P < 0.05$ compared with N group, $^{\#}P < 0.05$ compared with either Mod or NS group, $^{\&}P < 0.05$ compared with either TMP or SASP group

control group. The saline treatment group did not show any significant change in the expression of these proteins, while the TMP group as well as the SASP group demonstrated a dramatic decrease in the expression of C-MYC, iNOS and COX-2. Much more reduction in the expression is observed in the co-administration group.

TMP inhibits the activation of NF- κ B in cultured Caco-2 cell lines

Caco-2 cells were pretreated with TMP (40 μ g/ml) for 24 h and then exposed to LPS (100 ng/ml) for 6 h. LPS promoted the NF- κ B translocation into nucleus of Caco-2 cells as indicated by redistribution of protein level of NF- κ B P65, which was decreased in cytoplasm and increased in nucleus, compared with the vehicle-treated group (Figure 4A). In contrast, inhibition of NF- κ B translocation by TMP was further sup-

FIGURE 4. TMP inhibited the stimulation of LPS in cultured cells. **A)** TMP significantly inhibited the nucleus translocation of NF- κ B P65 that induced by LPS, Caco-2 cells were pretreated with TMP (40 μ g/ml) for 24 h and then exposed to LPS (100 ng/ml) for 6 h; **B)** TMP inhibited the secretion of TNF- α , IL-6 and IL-8 induced by LPS in Caco-2 cells, * P <0.05 as compared with control group, # P <0.05 as compared with LPS group; **C)** TMP inhibited the production of INF- γ and NF- κ B P65 induced by LPS in Th1 cells, as determined by ELISA and Western blot, * P <0.05 as compared with control group, # P <0.05 as compared with LPS group; **D)** ROS was measured by H2DCF-DA fluorescence, TMP inhibited LPS-induced intracellular ROS production (left shift).

ported by the observation that 24 h of TMP pretreatment upregulated cytoplasmic NF- κ B P65 protein levels and decreased nuclear NF- κ B P65 protein levels.

Inhibition of inflammatory factors and ROS production in cultured cells

Further measurements were performed to investigate whether TMP could exert an inhibition on NF- κ B downstream proinflammatory factors in colonic cells after stimulation with LPS. The cells were pretreated with TMP (40 μ g/ml) for 24 h and then treated with LPS (100 ng/ml) for 6 h. The results obtained in these assays showed that TMP significantly reduced

the production of different cytokines induced by LPS (Figure 4B). In LPS-stimulated Caco-2 cells, the production of TNF- α , IL-6 and IL-8 was significantly increased by LPS, while TMP significantly reduced the secretion of TNF- α , IL-6 and IL-8 into culture medium induced by LPS.

T helper cell 1 (Th1) is the host immunity effector against intracellular bacteria and protozoa. Th1 are triggered by IL-12, IL-2 and their effector cytokine, INF- γ . Th1 also plays an important role in colitis. As shown in Figure 4C, the INF- γ was significantly stimulated by LPS, and the pretreatment of TMP reduced the increase of INF- γ induced by LPS. The expression of INF- γ was also detected by Western blot, which showed the

similar results. TMP also inhibited the nuclear translocation of NF- κ B P65, as demonstrated by the increase of P65 in cytoplasm and decrease of P65 in the nucleus, which inhibited the activation of P65 by LPS.

The ROS production can be induced by the activation of NF- κ B, which is also involved in the development of colitis. Accordingly, the possibility that TMP modulates LPS-induced intracellular ROS formation in Caco-2 cells was examined. The results showed that TMP pretreatment significantly inhibited LPS-induced intracellular ROS formation in Caco-2 cells (Figure 4D).

Discussion

Recently, substantial advances in the understanding of the molecular pathogenesis of inflammatory bowel disease (IBD) have been made [19]. First, IBD has been found to be the most tractable of complex disorders for discovering susceptibility genes, which are related to epithelial barrier function, and innate and adaptive immunity. Second, efforts directed towards the identification of environmental factors implicate commensal bacteria (or their products) as drivers of IBD. Third, murine models, which exhibit many of the features of ulcerative colitis, have helped unravel the pathogenesis/mucosal immunopathology of IBD. Thus, IBD is a complex disease that includes the microbial–innate-immune-response interaction during the transition from physiological to pathological intestinal inflammation. Currently, the therapeutic efficiency is still limited due to the complexity of IBD; thus, finding an efficient way to treatment of IBD is still urgently needed.

In traditional oriental medicine, *Ligusticum wallichii* Franchet (*Ligusticum chuanxiong*) is widely used for the treatments of many ischemia disorders such as neurovascular and cardiovascular diseases. Tetramethylpyrazine (TMP), a purified and chemically identified component of chuanxiong, can prevent lipid peroxidation by scavenging highly reactive free radicals. It has been shown that TMP can alleviate kidney and brain damage by scavenging free radicals [20-21]. TMP had been used for the treatment of ulcerative colitis in China; however, its molecular mechanism has not been fully explored. In this study, TMP was found to reduce the colitis induced by oxazolone in a modified oxazolone-induced colitis mouse model, and the combination of TMP with SASP had a synergistic effect, suggesting that these two compounds could be used together for the treatment of colitis.

As NF- κ B has dual effect on colitis: NF- κ B is important to resolve inflammation in a controlled state, which as a barrier separating luminal bacteria and immune cells, and as an immunological barrier producing antimicrobial peptides and cytoki-

nes. NF- κ B is also a pivotal protein involved in proinflammatory signal transduction. The activation of NF- κ B has been demonstrated in colonic epithelial cells and macrophages of patients with IBD [22-23]. Numerous and diverse stimuli can induce NF- κ B activity. Typical inducers of NF- κ B include cytokines, such as tumour necrosis factor (TNF), interleukin1 (IL1) and viral and bacterial products, such as lipopolysaccharide (LPS) and cellular stress. NF- κ B p65 antisense oligonucleotide treatment was reported to be beneficial to experimental colitis [24].

To explore the molecular mechanisms, the effect of TMP on NF- κ B activity was investigated. TMP was found to inhibit the nucleus translocation of NF- κ B, and suppress its downstream pathways, such as iNOS, COX-2 and C-MYC, which play a critical role in the inflammation and cell proliferation. The co-administration of TMP and SASP dramatically inhibited NF- κ B pathway. These results indicated that the inhibition on NF- κ B pathway is critical for the pharmacological effect of TMP.

Further, the colonic cell line, Caco-2, was used as a cellular model by pretreating with TMP and then exposing the cells to LPS. The results showed that TMP significantly inhibited the nucleus translocation of NF- κ B induced by LPS, and its downstream proinflammatory factors, such as TNF- α , IL-6 and IL-8 were also decreased. ROS plays an important role in the pathogenesis of colitis, which is also decreased by TMP, demonstrated the anti-oxidative stress effect of TMP. As T helper cell 1 is involved in colitis, thus the effect of TMP on Th1 was also investigated at cellular level. The result showed TMP could inhibit the Th1 activation, as reflected by decreased INF- γ production and NF- κ B activation. These results suggested TMP has an anti-inflammatory effect in colitis that is induced by LPS.

In conclusion, this study showed TMP could alleviate the colitis induced by oxazolone, which is related to the inhibition of NF- κ B pathway, and suppress downstream signaling, via iNOS, COX-2, C-MYC and cytokine production, as well as decrease ROS production. The combination of TMP and SASP has a synergistic effect in animal models, suggesting that the combination may be beneficial for clinical treatment.

Acknowledgments

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Supplementary Material

TABLE 1. Disease Activity Index (DAI) scoring system

Score	Weight loss (%)	Stool consistency	Hematochezia
0	None	normal	(-)
1	1~5		
2	6~10	loose	occult blood(+)
3	11~15		
4	>15	watery	fecal blood(+)

TABLE 2. Histology scoring system

Feature	Score	Histology
Ulcer	0	none
	1	erosion
	2	ulcer
Lymphocytes infiltration	0	none
	1	mild
	2	moderate
	3	severe
Depth of infiltration	0	none
	1	submucosal
	2	muscle layer
	3	transmural
Neutrophils infiltration	0	none
	1	mild
	2	moderate
	3	severe
Depth of infiltration	0	none
	1	submucosal
	2	muscle layer
	3	transmural
Fibrosis	0	none
	1	mild
	2	moderate
Recess	0	normal
	1	spot disappear
	2	local disappear
	3	large disappear
Mucoprotein reduction	0	none
	1	mild
	2	moderate
	3	severe
Edema	0	none
	1	appear
Hemorrhage	0	none
	1	appear
Form pseudomembrane	0	none
	1	appear