J Huazhong Univ Sci Technol［Med Sci］
DOI 10.1007/s11596-008-0409-x 28 (4):415-420,2008

Oxymatrine Improves TNBS-induced Colitis in Rats by Inhibiting the Expression of NF-κB p65*

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Summary：Inflammatory bowel disease is thought to be regulated by the balance between Th1 and Th2 cytokines secreted by T cells, and NF-κB p65 also plays a predominant role in the intestinal inflammation. We evaluated the potency of oxymatrine, one of active components of Sophora Root, in inhibiting the immune responses and inflammation in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. The inflammation was markedly ameliorated in the oxymatrine-treated rats. The level of IL-2 was increased and that of IL-10 was decreased in colon tissue in the rat model, which was reversed by the treatment of oxymatrine. Moreover, the elevated expression of NF-κB p65 in colon tissue in the model was also improved by oxymatrine treatment. Our results suggest that oxymatrine might be beneficial for the abnormal immune responses and inflammation by regulating the unbalance of Th1 and Th2 cytokines secretion and inhibiting the expression of NF-κB p65 in colon tissue.
Key words：colitis; oxymatrine; interleukin 2 (IL-2); interleukin 10 (IL-10); nuclear factor-κB p65

Inflammatory bowel disease (IBD) is thought to result from inappropriate and ongoing activation of the mucosal immune system driven by the presence of normal luminal flora[1-3]. Patients typically present with bloody diarrhoea, passage of pus, mucus, or both, and abdominal cramping during bowel movements[4]. Though the highest incidence and prevalence of IBD have been reported to stabilise, the rates continue to rise in low-incidence areas such as southern Europe, Asia, and most developing countries[5]. In recent years, advances in the immunology of IBD have led to new therapeutic concepts, such as modulation of interleukin-10 (IL-10), blockade of tumor necrosis factor (TNF), blockade of T cells, blockade of inflammatory cell migration and adhesion and so on. Most of them, however, are associated with important side-effects[6-10].

NF-κB is a transcription factor which regulates the expression of a variety of genes that encode proinflammatory cytokines and proteins of innate immunity and acute phase response[11-12]. Its family includes so far NF-κB-1 (p50 and its precursor p105), NF-κB-2 (p52 and its precursor p100), p65 (RelA), c-Rel (Rel), and RelB. In IBD patients, the lamina propria macrophages have been found to increase NF-κB p65 expression and DNA-binding activity with an increased production of TNF-α and other cytokines[13]. Furthermore, in the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model, NF-κB p65 is also highly expressed and the acute inflammation could be abrogated by blocking NF-κB p65 expression with an antisense oligonucleotide targeted against the translation start site[14]. Therefore, the blockade of NF-κB p65 subunit might be a new therapeutic strategy for the treatment of IBD.

Oxymatrine, one of active components of Sophora Root, has been reported to be effective in the treatment of colitis, mainly by down-regulating the NF-κB activation and some other inflammatory cytokines[15]. Nevertheless, it is still uncertain whether the down-regulation is associated with the NF-κB p65 subunit. In this article, we examine the ability of oxymatrine to inhibit the expression of NF-κB p65, thereby reducing the level of IL-2 and increasing IL-10 in the colon tissue in TNBS-induced colitis rat model.

1 MATERIALS AND METHODS

1.1 Animals and Treatment

Male Sprague-Dawley (SD) rats weighing 180–225 g were purchased from the Animal Center of Tongji Medical College, Huazhong University of Science and Technology (HUST), Wuhan, China (No. SYXK(Er) 2004–0028), and maintained with food and water available freely at the specific pathogen-free (SPF) Animal Center of Tongji Medical College, HUST, Wuhan, China.

All rats were randomly divided into four groups after a 1-week period of adaptation to surroundings: nor-

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*This study was supported in part by a grant from Post-doctoral Sciences Foundation of China (No. 2005037679) and a research grant from National Natural Sciences Foundation of China (No. 30772878).

mal control group (n=10) receiving food and water freely without any treatment, a normal saline group, a mesalazine group and an oxymatrine group, with 10 animals in each of the group. There was no significant difference in the body weight among the four groups. Twenty-four hours after TNBS administration (for making clotitis model), rats in the normal saline group were orally given 0.9% normal saline. The rats in the mesalazine group were orally given mesalazine (Ethypharm Pharmaceutical Co. Ltd., France, No. 03832) at 0.42 $g \cdot kg^{-1} \cdot d^{-1}$ dissolved in distilled water, and diluted with water to appropriate concentration. The rats in the oxymatrine group were injected intramuscularly with oxymatrine (Tianjin Biochemical Pharmaceutical Co. Ltd., China, Batch no. 20061201) at 63 $mg \cdot kg^{-1} \cdot d^{-1}$, which was based on the data of previous report[15]. Fifteen days after the treatment, all rats, after 1-day fasting, were sacrificed. Ethical approval for this study was obtained from the Animal Experimental Committee of HUST, Wuhan, China.

1.2 Induction of Colitis by TNBS

TNBS was obtained from Sigma, USA (Batch no. P2297). The rat model of colitis was induced by using the method described by Morris *et al*[16-17] with minor modifications. After feeding on fasting diet for 24 h, rats were anesthetized with 2% pentobarbital sodium by intraperitoneal injection to induce colitis. A 15-cm rubber catheter with an inner diameter of 2 mm was inserted 8 cm into colon from anus. An ethanol solution (50%, 0.25 mL) containing 100 mg/kg TNBS was injected into the colon lumen via a gauge needle. The rats were maintained in a inverted position for 30 s and was then switched to a supine Trendelenberg position until they recovered from anesthesia to prevent leakage of TNBS.

1.3 Histopathological Examination

The rats were fixed on an operation table under anesthesia by 2% pentobarbital sodium. Whole colon was taken out and cut along the longitudinal axis. After flushed with 0.9% saline water, the colon was tiled on a sterile virgin paper to evaluate the degree of intestinal injury. The extent of mucosal damage was assessed using the colon macroscopic scoring system reported by Wallace *et al*[18]. Ulceration (number in the brackets denote points): focal hyperemia, no ulcer (1); ulceration, no hyperemia/bowel wall thickening (2); ulceration, inflammation at one site (3); ulceration, inflammation at 2 or more sites (4); major injury > 1 cm, 6–10 major damage > 2 mm (5). Adhesion: minor (colon easily separated from other tissue) (1); major (2). Diarrhea: bowel wall thickening (1). Strips of colonic tissue (15–30 mg from segments most intensively affected by the inflammation) were cut out, immersed in liquid nitrogen, and kept at −80°C until the RNA extraction and enzyme-linked immunosorbent assay (ELISA). Another part of colon tissue was immediately fixed for 24 h in 4% paraform for paraffin embedding and tissue staining. Two transverse sections (4–6 μm thick) were HE-stained and examined under the light microscope to assess the mucosa injury by an experienced pathologist who had no knowledge of the study design.

1.4 Evaluation of the Concentration of IL-2 and IL-10 in Colon Tissue by ELISA

Part of the frozen colon tissue was cut into pieces and homogenized in the 0.9% saline water. The supernatant of the homogenate was collected for the determination of the concentrations of IL-2 and IL-10 by ELISA 20 min after centrifugation at 4000 r/min. IL-2 and IL-10 ELISA kits (Jingmei Biotech Co., China) were used for the measurement by following the manufacturer's instructions.

1.5 Immunohistochemical Assay

To visualize the presence and localization of NF-κB p65 within the colon, immunohistochemical studies were performed by using a K75619A kit (Beijing Zhong Shan-Golden Bridge Biological Technology Co. Ltd., China). To minimize the background staining, all sections were first blocked with normal goat serum for 15 min at room temperature. Then the slides were incubated with an antibody directed against rat NF-κB p65 (#D0907, Santa Cruz Biotechnology Inc., USA). Sections were counterstained with hematoxylin. The positive expression presented yellow or brown reactants in cytolymph and nuclei under light microscope. In the visual field of 400-fold-magnification, positively stained cells were counted to determine the ratio of positive cells out of 100 randomly selected cells.

1.6 Expression of IL-2, IL-10, NF-κB p65 mRNA in Colon Tissue by Real-time Polymerase Chain Reaction (Real Time-PCR)

RNA extraction for another part of frozen colon tissue was performed in accordance with the instructions of the manufacturer (RNeasy Mini Kit, QIAGEN Inc., Japan). Reverse transcription with up to 4 μg of total RNA was carried out in a total volume of 20 μL containing 250 pmol of random primer, 100 U of Super-Script Ⅱ RNase H-reverse transcriptase (Invitrogen, USA) in 50 mmol/L Tris-HCl (pH 8.3), 40 mmol/L KCl, 6 mmol/L $MgCl_2$, 1 mmol/L DTT, and 10 mmol/L dNTPs. Initially, total RNA solution mixed with random primer was heated at 70°C for 10 min and immediately chilled on ice, and then the other reagents were added. First-strand cDNAs were obtained after 50 min reaction at 42°C and after 5 min at 98°C. Power SYBR Green PCR Master Mix (Applied Biosystems Inc.) was used. IL-2 sense primer: 5'-CAGGTGCTCCTGAGAGGGATCG-3'; antisense primer: 5'-GAGCCCTTGGGGCTTACAAAAAG-3'; amplification product: 504-bp cDNA. IL-10 sense primer: 5'-GCTCAGCACTGCTATGTTGC-3'; antisense primer: 5'-TTCATGGCCTTGTAGACACC-3'; amplification product: 469-bp cDNA. NF-κB p65 sense primer: 5'-GAAGAAGCGAGACCTGGAG-3'; antisense primer: 5'-TCCGGAACACAATGGCCAC-3'; amplification product: 330-bp cDNA. GAPDH sense primer: 5'-TATTGGGCGCCTGGTCACCA-3'; antisense primer: 5'-CCACCTTCTTGATGTCATCA-3'; amplification product: 752-bp cDNA. All of the primers were provided by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd., China. Quantification of target cDNA and an internal reference gene GAPDH was performed in 96-well plates on the ABI PRISM7700 Sequence Detection System (ABI, USA). Data collection, and analyses were carried out by using a software package that came with the machine. The PCR was performed in a final volume of 25 μL containing cDNA template 2.5 μL, sense primer and antisence primer 0.25 μL, respectively, SYBR Green PCR Master Mix 12.5 μL and DNA water 9.5 μL. Each sample was analyzed in

triplicate. Thermal cycler conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The comparative CT method of data analysis was used to analyze the data. CT is the PCR cycle at which an increase in reporter fluorescence above the baseline level was first detected. CT of target gene and CT of internal reference gene were calculated for each sample. ΔCT was the difference in CT between target gene and reference gene. ΔΔCT was the difference in ΔCT between sample and calibrator sample. The expression amount of target gene, normalized to an internal reference and relative to calibrator, was calculated by: 2-ΔΔCT.

1.7 Statistical Analysis

Data were expressed as $\bar{x}\pm s$. Statistical analysis of data was done by using one-way analysis of variance (ANOVA) for evaluating differences in the macroscopic damage scores, concentration of IL-2 and IL-10, ratio of NF-κB p65-positive cells and the expression of mRNA in colon tissue. When a significant difference was found between the items of a specific factor, multiple comparisons were made by using the Bonferroni test, in order to identify which items presented differences between one other. A P <0.05 was considered to be statistically significant.

2 RESULTS

2.1 General Health State and Survival

The general state of rats in the control group was normal, including body weight, stool condition, appetite and activity. Three days after injection of TNBS into the colon lumen, rats in the model group and two treatment groups developed diarrhea, bloody purulent stool and some of them was attached on the crissum, decreased appetite and loss of body weight, withered fur and lack of activity. After the treatment with mesalazine or oxymatrine for 15 d, the stool became normal and the appetite was increased, especially in the oxymatrine treatment group. While the stool of rats in the model group was still bloody and purulent and the body weight was decreased gradually (table 1).

Table 1 Concentrations of IL-2 and IL-10 and percentage of NF-κB p65 positive staining cells in colon tissue ($\bar{x}\pm s$)

Groups	n	Body weight	IL-2 (pg/mL)	IL-10 (pg/mL)	NF-κB p65 (%)
Normal group	10	240.3±16.9**	30.44±12.03**	58.92±3.70**	9.57±4.31**
Model group	8	194.9±18.5	231.48±40.78	18.64±0.65	43.05±13.80
Mesalazine Group	10	215.8±19.2*	110.44±49.59*	53.05±1.85*	17.20±6.54**
Oxymatrine group	10	227.4±18.8**	102.93±21.10*	50.13±1.40*	16.02±7.27**

IL: interleukin; NF-κB: nuclear factor κB
*P<0.05, **P<0.01 compared with model group

Two rats died on the 9th and 11th day after the injection of TNBS in the model group and no rat died in other groups. Ankylenteron and enterostenosis were observed in the distant colon and colectasia and thickening of colonic wall were noted in the proximal colon in two dead rats at autopsy.

2.2 Histopathological Findings

Macroscopic damage scores of rats in the model group were significantly higher than those of rats in the normal control group. Treatment with mesalazine or oxymatrine significantly decreased the macroscopic damage scores in comparison to untreated colitic rats. Animals in mesalazine group and oxymatrine group showed no significant difference in macroscopic damage scores (fig. 1).

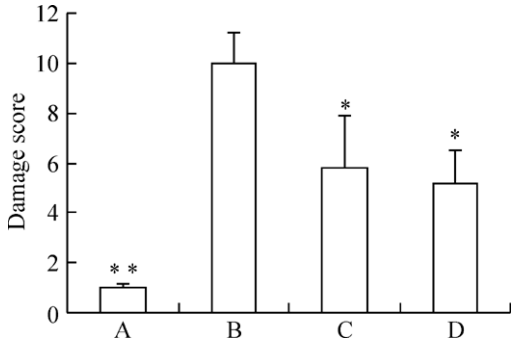

Fig. 1 Macroscopic damage scores of colon tissue
A: normal control group; B: model group; C: mesalazine treatment group; D: oxymatrine treatment group
*P<0.05, **P<0.01 compared with model group

On the HE-staining slides, damaged mucosa muscular layers and glands by ulcer together with reduced goblet cells were observed in untreated colitic rats (fig. 2A). In the mesalazine group, anabrotic mucosa, hydropic and congestive submucosal layers and infiltration of large amount of neutrophils and plasma cells in proper layer were observed (fig. 2B). In the oxymatrine-treated rats, increased goblet cells and decreased infiltrating inflammatory cells were noted (fig. 2C).

2.3 Concentration of IL-2 and IL-10 in Colon Tissues

Significantly increased IL-2 and decreased IL-10 levels were found in the model rats in comparison with normal controls (P<0.01). After the treatment with mesalazine or oxymatrine, the concentration of IL-2 was reduced, while the concentration of IL-l0 was elevated (P<0.05, respectively). Moreover, no significant differences in the concentration of IL-2 and IL-10 were found between two treatment groups (P>0.05) (table 1).

2.4 Immunohistochemical Findings

NF-κB p65 was mainly expressed in the nucleus and cytoplasm of epithelia and macrophages. Its expression was strong in the model group (fig. 3A) but it was weaker in the normal control group (fig. 3D). In two treatment group, some nucleus and cytoplasm were stained as pale yellow (fig. 3B and 3C). The percentage of positive staining cells was significantly higher in the model group than other three groups respectively (P<0.01). No significant difference was indicated be-

tween two treatment groups (P>0.05) (table 1).

2.5 Colonic NF-κB p65 mRNA Expression

As shown in fig. 4, colonic expression of IL-2 and NF-κB p65 mRNA was significantly increased in the model group and decreased after the treatment of mesalazine or oxymatrine (P<0.01 or 0.05). The expression of IL-10 mRNA was reduced in the model group and elevated in the two treatment groups significantly (P<0.01 or 0.05). However, there was not significant difference between two treatment groups (P>0.05).

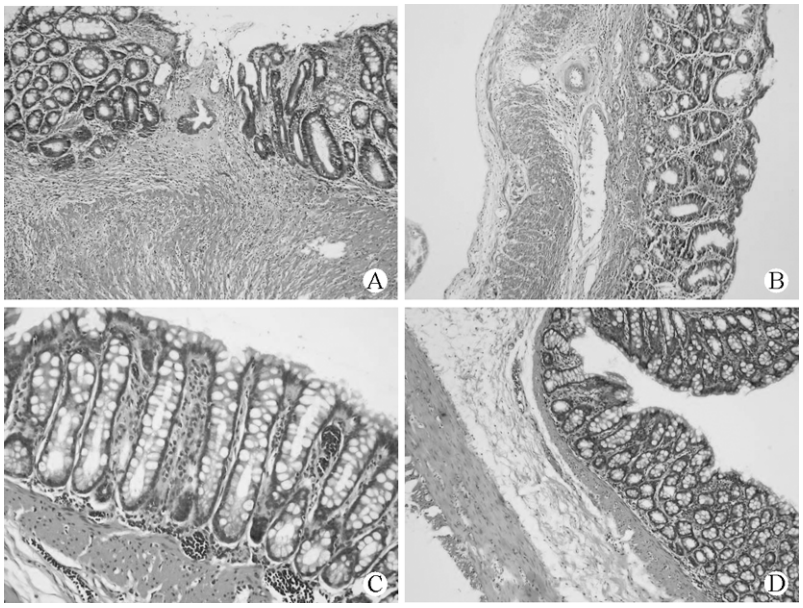

Fig. 2 The pathological examination of the colon tissue (HE×100)
A: the mucosa muscular layer and glands damaged by ulcer together with few goblet cells observed in model rats; B: the anabrotic mucosa, hydropic and congestive submucosal layer and large amount of neutrophils and plasma cells infiltration in proper layer in the mesalazine treatment group; C: increased goblet cells and decreased inflammatory cell infiltration in the oxymatrine-treated rats; D: the normal colonic mucosa

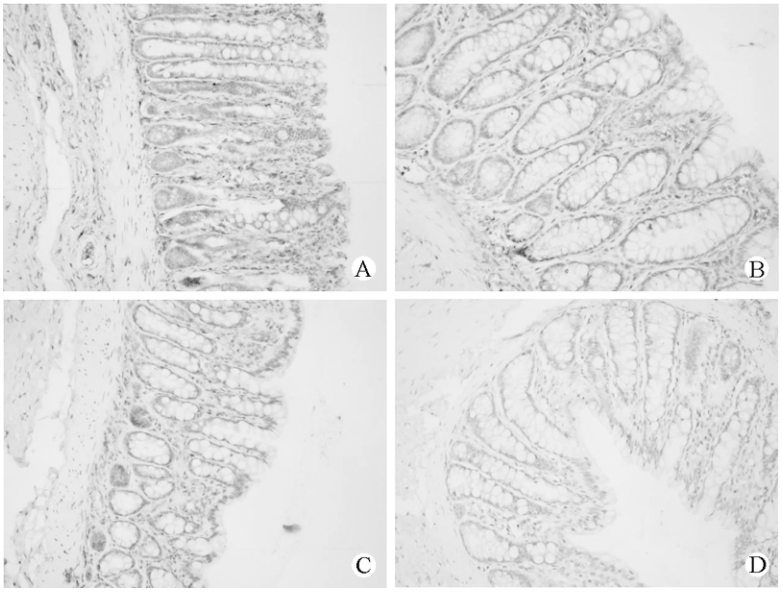

Fig. 3 The immunohistochemical findings of NF-κB p65 in four groups (SP×200)
A: the strongly positive expression in the model group; B: pale yellow stained nucleus and cytoplasm in the mesalazine treatment group, similar to C (oxymatrine treatment group); D: slightly positive expression the normal colonic mucosa.

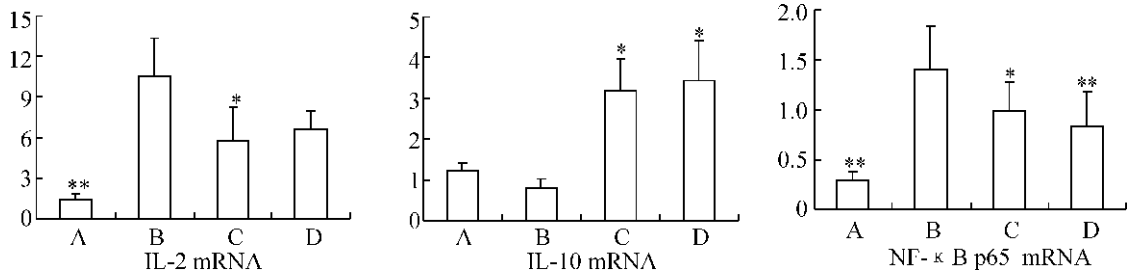

Fig. 4 The mRNA expression of IL-2, IL-10 and NF-κB p65 in four groups
A: normal control group; B: model group; C: mesalazine treatment group; D: oxymatrine treatment group
$^{*}P<0.05$, $^{**}P<0.01$ compared with model group

3 DISCUSSION

This study showed that treatment with oxymatrine, the active component of sophora root, significantly reduced colonic ulcer, inflammation and cellular infiltration in TNBS-induced colitis. Oxymatrine also modulated the balance of Th1 and Th2 cytokines and inhibited the expression of NF-κB p65 in colon tissue in colonic rat model.

TNBS mixed with ethanol is one of most widely used methods to induce murine colitis. The ethanol acts as the destroyer of the mucosal barrier. The breakage in the mucosal barrier leads to increased exposure of the mucosal immune system to the gut microflora, and thus giving the hapten in TNBS (trinitrophenyl) opportunity to modify the autologous molecules in the mucosa and result in priming of antigen-specific T cells. TNBS-induced colitis is used to study a Th1-driven disease mimicking human IBD, especially Crohn's disease[19]. The percentage of ethanol varies between 35% and 50%[20]. In this model, 50% of ethanol was used and we observed the classical histopathological changes in colon tissue, such as infiltration of neutrophils and macrophages into the colonic mucosa and submucosal layers, thickening of the colon wall, loss of goblet cells, and so on, which were in consistency with previous study[21]. Moreover, the change in the levels of IL-2 and IL-10 was in line with Th1-driven colitis.

T lymphocytes play a central role in the intestinal immune system[22]. Recent studies suggested that the balance between Th1 and Th2 cytokines secreted by T cells appeared to regulate IBD[23]. IL-2 is produced by activated Th1 lymphocytes and stimulates the immune response mediated by the macrophages, natural killer (NK) cells and cytotoxic T cells. The presence of large number of activated T cells in the involved mucosa of IBD patients suggests that IL-2 might be involved in the induction of inflammation. Moreover, it is generally accepted that IL-2 level is increased in active Crohn's disease[24]. IL-10 is produced by Th2 lymphocytes and acts to inhibit macrophages and other antigen-presenting cells. It also inhibits cytokines produced by Th1 lymphocytes in response to these APC. The important regulatory role of IL-10 in the gut became more obvious when mice with IL-10 deficiency, made by gene-knock-out, developed chronic enterocolitis[25]. Based on the previous experimental findings in animal models of intestinal inflammation, IL-10 therapy was shown to be very promising as a new anti-inflammatory therapy for Crohn's disease[26]. In this study, we found that the level of IL-2 and its mRNA expression were increased and IL-10 was decreased in the TNBS-induced colitic model, which might be important mechanisms reponsible for the intestinal inflammation. In addition, treatment with oxymatrine was capable of reducing the level of IL-2 and its mRNA expression, and elevating those of IL-10. Hence, oxymatrine might be an effective agent for the regulation of the Th1 and Th2 secretion.

The NF-κB family includes key transcription factors of lymphocytes and macrophages, that participate in immune responses and inflammation[11]. The functional importance of NF-κB in inflammation lies in its ability to regulate the promoters of a variety of genes whose products, such as cytokines, adhesion molecules and acute phase proteins, are critical for inflammatory processes[27-28]. Subunit p65, also named RelA, is one of the five members of NF-κB family. Neurath *et al*[14] demonstrated that NF-κB p65 was a predominant factor in the intestinal inflammation in TNBS-induced colitis. In our study, NF-κB p65 expression was increased, which was accompanied by increased production of proinflammatory cytokines, such as IL-1, IL-6 and TNF-α, suggesting they played very important roles in the development of inflammation of IBD. Hence, inhibiting the expression of NF-κB p65 was recommended as one of effective approaches for the treatment of IBD[14]. Interestingly, IL-10 in the normal gut may modulate the activity of NF-κB thereby indirectly affecting the expression levels of IL-1, IL-6 and TNF-α[29-36]. Moreover, NF-κB p65 mediates transcriptional activation of the IL-2 gene and regulates its expression[23]. Oxymatrine was found to reduce the expression of NF-κB p65 mRNA and the NF-κB p65 protein in this study. On the basis our finding about IL-2 and IL-10 as mentioned above, we are led to conclude that oxymatrine is capable of decreasing the level of IL-10, inhibiting the expression of NF-κB p65 and increasing the level of IL-2 in colon tissue.

To sum up, the colonprotective effect of oxymatrine, like mesalazine, may be due to its inhibition of the NF-κB p65 expression and modulation of the balance of Th1 and Th2 cytokines.

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(Received May 8, 2008)